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CII. THE ENZYMIC PRODUCTION OF VOLATILE PRODUCTS FROM NICOTINE UNDER THE INFLUENCE OF TOBACCO-LEAF EXTRACTS.

(A REPLY TO A. FAITELOWITZ.)

By ANDOR FODOR and ADOLF REIFENBERG.

From the Institute of Biological and Colloidal Chemistry of the Hebrew University, Jerusalem.

(*Received April 6th, 1927.*)

A paper has been published in this *Journal* by Faitelowitz [1927] dealing with experiments from which it may be concluded that mixtures of fermented cigarette-tobacco with water are infected by bacteria in the open air; at the same time an alkaline reaction develops, which is not the case if chloroform is applied. Probably no one will doubt that these facts are correct.

We wish to point out, however, that in our experiments [1925] the macerates were prepared "under the usual conditions of sterility" (p. 830), *i.e.* by using toluene and thymol, and, nevertheless, decomposed nicotine.

The formation of acids during tobacco fermentation was not denied by us; on the contrary the formation of carbonic acid during the decomposition of nicotine has been noted (p. 831).

Since the publication of our paper in this *Journal* we have reported on many additional experiments with regard to the question of tobacco fermentation [1926]. We found a strong parallelism between the decomposition of nicotine and the amount of oxygen absorbed by moistened tobacco leaves, which is a further argument in support of our view that the reaction is one of oxidation. Furthermore, we found an oxygen absorption even after filtering the macerates through porous clay filters or after adding germicides, *e.g.* vucine, mercuric chloride, and even potassium cyanide. Neither the respiration, nor the decomposition of nicotine connected with it, is in any way associated with bacterial processes. Our views on the sum-total of the fermentation process are presented in the above-mentioned paper.

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CIII. THE INTERACTION OF A FINELY DIVIDED LEAD SUSPENSION WITH BLOOD-SERUM, RINGER SOLUTION, AND AQUEOUS PHOSPHATE SOLUTION.[1]

By JOHN BROOKS.

From the Muspratt Laboratory of Physical Chemistry and Electrochemistry, University of Liverpool.

(*Received May 17th, 1927.*)

The present paper deals with the changes which take place when lead in a fine state of subdivision in a stabilising medium (as used in the treatment of malignant disease) is added to blood-serum.

The chief points on which information was desired were:

(i) The possible production of the toxic ionic lead after injection of the lead suspension into the blood-stream.

(ii) The chemical reactions between the constituents of the suspension and those of the blood.

In the literature dealing with lead poisoning there are several references to the form which lead compounds assume in the blood, but no very definite conclusions have been reached.

The question has been investigated by Dr M. Jowett in this laboratory from the standpoint of the solubility of inorganic lead salts in water and in serum respectively. Of these salts the most important are the tertiary and secondary phosphates and the carbonate. Jowett has calculated that the tertiary phosphate $Pb_3(PO_4)_2$ is, under body conditions, the least soluble. Lead salts when introduced into the blood should therefore ultimately pass into this compound since there are phosphate ions present in serum. This has been suggested by Fairhall *et al.* [1926].

Though the transformation into the phosphate would probably be rapid when soluble lead salts were introduced into the blood, the same might not be the case when the lead was in a colloidal form. Various measurements of the rate of combination have therefore been made.

Because of the complexity of both lead suspension and serum some work has also been done with aqueous solutions of sodium phosphate and with a modified Locke-Ringer solution.

[1] This investigation was undertaken on behalf of the Liverpool Medical Research Organisation.

Experimental.

The lead suspension. This is prepared by Bredig's electrical sparking method using lead electrodes in a 0·5 % gelatin solution containing calcium chloride ($N/200$) as stabilising medium. The average diameter of the particles in the black suspension after centrifuging is $0{\cdot}2\mu$, these particles being negatively charged. The freshly-prepared suspension contains approximately 0·5 % total lead; a typical suspension had the composition: 0·51 g. total Pb per 100 cc. This contains

65·6 % Pb as metallic lead,
8·9 % Pb as lead carbonate,
25·5 % Pb as lead hydroxide[1] (by difference).

The concentration of ionic lead (determined by colorimetric estimation of lead in the ultra-filtrate from the suspension) is *ca.* 0·009 %. This corresponds approximately with the value expected from the solubility of lead hydroxide[2]. The chemical composition of the particles varies with the time and extent of exposure of the suspension to the atmosphere, and the suspension eventually turns white on complete oxidation of the lead to lead hydroxide. The total lead content of each fresh suspension used was estimated by the iodometric method described by Fairhall *et al.* [1926].

The aqueous phosphate solution. The solution used had the following composition: 0·00156 *M* Na_2HPO_4, 0·00095 *M* HCl, 0·9 g. NaCl per 100 cc. The p_H of this solution was 7·4 and 1 cc. contained 0·0484 mg. phosphorus, which is within the range of the inorganic phosphorus content of blood-serum.

Ringer solution[3]. This solution was prepared by mixing isotonic solutions of the various salts and had the following composition:

Salt	Moles per litre
NaCl	0·137
KCl	0·00280
$CaCl_2$	0·00197
$NaHCO_3$	0·0264 (unless otherwise stated)
Na_2HPO_4 / KH_2PO_4	0·00176 „ „

This solution is used in conjunction with a gas mixture of 95 % oxygen and 5 % carbon dioxide. When saturated with this mixture the p_H at 37·5° is 7·49. The p_H is given by the Henderson-Hasselbalch equation [1917]

$$p_H = p_K + \log \delta + \log \frac{[NaHCO_3]}{[CO_2]},$$

[1] For the sake of convenience the compound formed by the oxidation of metallic lead in contact with water is called lead hydroxide and written $Pb(OH)_2$, although according to Pleissner [1907] it has the composition $Pb_3O_2(OH)_2$; Glasstone [1922] gives a more complex composition to this substance.

[2] Pleissner [1907] gives 100·5 mg. PbO per litre at 18° for the solubility of lead hydroxide in water.

[3] This solution (with the exception of the phosphate present) as also the method of its p_H regulation is similar to that used by Warburg [1924] in his work on glycolysis.

where $[NaHCO_3]$ and $[CO_2]$ are the concentrations in moles per litre of (sodium) bicarbonate and dissolved carbon dioxide respectively. In the present case at 37·5°

$$[CO_2] = 1{\cdot}18 \times 10^{-3},$$
$$[NaHCO_3] = 2{\cdot}64 \times 10^{-2},$$

whence

$$p_H = 7{\cdot}49,$$
$$p_K + \log \delta = 6{\cdot}14.$$

Serum. Fresh cow's serum was used, thymol being added as preservative. In order to maintain a physiological p_H the serum was saturated with the gas mixture referred to above and subsequent operations were conducted in presence of this mixture. The p_H was then known when the bicarbonate concentration of the serum was determined.

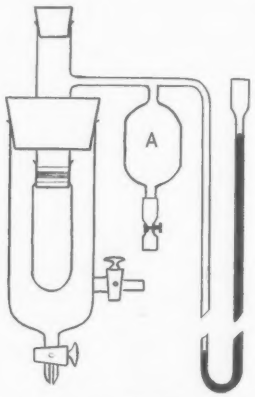

Fig. 1. Apparatus for ultra-filtration.

When the lead suspension was added to serum or to the other two solutions mentioned above there was usually no immediate aggregation of the particles. To estimate the amount of phosphate ion which had disappeared from solution it was necessary to remove the suspended particles. This was done by ultra-filtration under a pressure of 200–300 mm. Hg through collodion bags (prepared from a 6 % collodion solution). The arrangement represented in Fig. 1 permitted ultra-filtration in an atmosphere of the gas mixture and also at different temperatures. The collodion bag was secured to the glass tube by a strip of parchment and tightly-wound linen thread. The air space *A* was necessary to prevent a too rapid fall of pressure during filtration. If necessary the air was displaced by the gas mixture from the apparatus. The first 1·5 cc. of filtrate were rejected. With serum the rate of filtration was 3–5 cc. per hour, with Ringer solution this rate was approximately doubled.

Inorganic phosphorus was estimated by Briggs' modification [1924] of the Bell-Doisy colorimetric method. Bicarbonate concentrations were determined

(at 37·5° and in presence of the gas mixture) with Warburg's apparatus and technique [Negelein, 1925].

The experimental procedure was as follows. The lead suspension was added to serum or to Ringer solution previously saturated with gas mixture in such amounts that excess phosphate was present. The gas mixture was bubbled through the liquid for 3–4 minutes, ultra-filtered after a given time, and the uncombined phosphate in 1–3 cc. of the filtrate estimated. The gas mixture was not used with the aqueous phosphate solution.

The combination of the metallic lead present with phosphate is necessarily preceded by its transformation into the hydroxide[1]. Rough measurements had previously shown that the rate of this change in the suspension itself when exposed to air is dependent on the rate of stirring and the area of the air-liquid interface. The reaction is rapid given a sufficient supply of oxygen—as can be seen by the immediate whitening which takes place on adding the suspension to large enough volumes of water. With the amounts of suspension and serum, etc., used in the present experiments, the reaction was complete in less than 5 minutes[2].

Table I gives the results obtained when the lead suspension was added to aqueous phosphate solution. The mixtures were allowed to stand for 7 days before being filtered. The p_H of the mixtures varied from 8·2 to 8·8[3].

Table I. *Lead suspension contains* 0·517 % *total lead.*

cc. phosphate solution	cc. suspension	g. atoms phosphorus bound to lead × 10^4	g. atoms P bound / g. atoms Pb
80	1	0·156	0·62
40	1	0·158	0·63
40	2	0·321	0·64
40	3	0·470	0·63

The last column gives the ratio of g. atoms of phosphorus, which as phosphate had disappeared from solution, to g. atoms of total lead present. The value for the ratio does not differ greatly from that for tertiary lead phosphate, $Pb_3(PO_4)_2$, *i.e.* 0·67. The similarity of the results at different final concentrations of phosphate and also the large amounts of the latter which have disappeared do not favour the view that the disappearance is due to adsorption alone. It seems probable therefore that the particles are converted into particles of lead phosphate.

Before the interaction of the lead suspension with serum was investigated, a few filtration experiments with serum alone were carried out. The ultrafiltrate was colourless and protein-free and contained the same concentration

[1] Sackur [1905] has shown that pure lead is only attacked by dilute acids in the presence of oxygen. The velocity of solution is practically independent of the strength and concentration of the acid but dependent on the concentration of dissolved oxygen and the rate of stirring.

[2] Measured approximately by a manometric method.

[3] The change of p_H in the very weakly buffered system is apparently due to the following reaction:

$$2HPO_4'' + 3Pb(OH)_2 \rightarrow Pb_3(PO_4)_2 + 2H_2O + 4OH'.$$

of bicarbonate and inorganic phosphorus as the serum itself. Further, the concentration of inorganic phosphorus in the filtrate was unaltered after the serum had stood for 14 days.

Table II summarises the results obtained on adding the suspension to phosphate and Ringer solution and to serum. For comparison are added the values obtained when a solution of lead acetate (5 mg. Pb per cc.) was used in place of the suspension. In the latter cases the mixtures were usually filtered immediately; in the former after standing for several days. Similar results were obtained at room temperature and at 37·5°.

Table II.

	g. atoms P bound / g. atoms Pb		
	Phosphate solution	Ringer solution	Blood-serum
Lead suspension	0·62	1·0	1·1
Lead acetate	0·62	0·69	1·1

These values are each the average of several experiments[1] (3–7). The addition of small volumes of lead suspension to Ringer solution or serum does not change the p_H appreciably (the p_H of the mixture being calculated from the bicarbonate concentration of the ultra-filtrate)[2]. The decrease in bicarbonate concentration due to dilution by the suspension is balanced by the bicarbonate formed in the reaction, *e.g.*

$$Pb(OH)_2 + HPO_4'' + 2CO_2 \rightarrow PbHPO_4 + 2HCO_3'.$$

The ratios in Table II fall into two classes which correspond approximately with the ratios for the secondary phosphate $PbHPO_4$ and the tertiary phosphate $Pb_3(PO_4)_2$, *i.e.* 1·0 and 0·67 respectively. Assuming that the results represent the formation of these compounds, it follows that the secondary phosphate is formed when lead acetate or lead suspension is added to serum, whilst with Ringer solution lead suspension gives the secondary compound and lead acetate the tertiary. The tertiary phosphate is formed when either the suspension or lead acetate is added to aqueous phosphate solution. These differences can hardly be due to the effect of p_H since with the phosphate solution a ratio 0·62 was obtained both with lead acetate (p_H of mixture *ca.* 6·0) and lead suspension (p_H of mixture *ca.* 8·5), whereas with Ringer solution or serum (p_H of mixture *ca.* 7·5)[3] the two ratios, 1·0 and 0·67, were obtained. It seems preferable to attribute the formation of the secondary phos-

[1] The values of a given series (*e.g.* Table I) agreed well between themselves. Greater deviations (5 %) were obtained between the different series. This may have been due to the slight differences in tint sometimes obtained between the standard and the experimental solutions in the colorimetric method.

[2] With the mixture in Table IV, $[NaHCO_3]$ of mixture ultra-filtrate = 0·0266.

[3] Similar results were obtained with Ringer solution and serum in the absence of the gas mixture (p_H of mixture > 8·5).

phate under certain conditions to delayed equilibrium[1] even though the same ratios as those quoted above were obtained after the mixtures had stood for 14 days. It may be noted that the greater the complexity of the system the greater is the tendency to give the secondary phosphate.

Rate of combination of phosphate ions with the lead suspension.

The rate of combination was measured by filtering portions of the mixture at various time intervals and estimating the uncombined phosphate in 2 cc. of filtrate. The amount of suspension used was such as to combine with approximately two-thirds of the phosphate. Under the conditions employed the preliminary oxidation of metallic lead to lead hydroxide was complete before filtration was commenced.

Two series of experiments were carried out with Ringer solution and serum at 20° and 37·5°. The filter (with the exception of the manometer) was immersed in a thermostat. The results are given in Tables III and IV. The times are reckoned from the time of mixing to the mid-time of filtration.

Table III.

75 cc. Ringer solution.
4·5 cc. lead suspension = 20·7 mg. total Pb.

	g. atoms P bound / g. atoms Pb	
Time (hours)	20°	37·5°
0·5	0·92	0·96
1·5	0·93	0·96
3·0	0·95	0·98
5·0	0·95	0·97
8·3	0·98	0·98

Table IV.

50 cc. serum (0·00196 *M* phosphate).
3·0 cc. lead suspension = 13·8 mg. total Pb.

	g. atoms P bound / g. atoms Pb	
Time (hours)	20°	37·5°
2·0	0·83	0·95
6·0	0·86	0·98
25·0	0·95	0·99
50·0	0·97	1·04
73·0	1·04	1·00

When these values are plotted (cf. Fig. 2) the curves show a rapid rise in the amount of combined phosphate up to approximately 90 % of the final amount, followed abruptly by a much slower combination of the particles with the remaining 10 % of phosphate. The greater portion of the reaction is

[1] Holt, La Mer and Chown [1925], who have studied the solubility of the calcium phosphates in water, found under certain conditions where the tertiary salt was the least soluble that the secondary phosphate was initially formed and that the subsequent transformation into $Ca_3(PO_4)_2$ was slow.

too rapid to be investigated by the present method. It can be seen, however, that at 20° the rate of combination in serum is somewhat slower than in Ringer solution. At 37·5°, although the rates are similar, the degree of dispersion of the suspension in the two liquids is very different (see below).

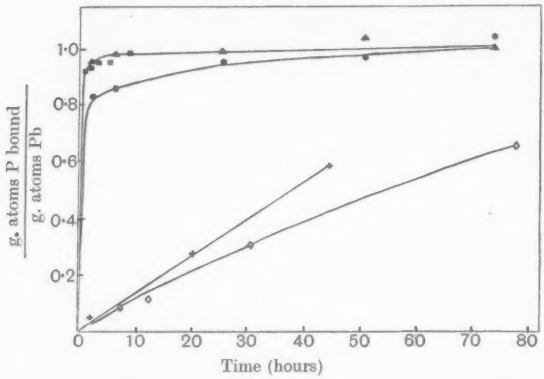

Fig. 2.

▲ Serum and lead suspension at 37·5°. • Serum and lead suspension at 20°.
■ Ringer solution and lead suspension at 20°. ◇ + Serum and lead carbonate suspension at room temperature.

The rates in serum and Ringer solution were compared under more comparable conditions by preparing a Ringer solution containing the same concentrations of bicarbonate and of phosphate as were contained in the serum. These were 0·0312 and 0·00204 *M* respectively. The results are given in Table V.

Table V.

Room temperature.
50 cc. serum or Ringer solution.
3 cc. lead suspension = 16·2 mg. total Pb.

Time (hours)	g. atoms P bound / g. atoms Pb: Ringer	Serum
1·5	0·92	0·80
7·0	0·92	0·83
8·0	0·93	0·86
26·0	0·96	0·90

The slower rate in serum is probably due to the presence of a film of serum proteins round the particles of the suspension.

It was thought that the rapid decrease in the rate of combination might be accounted for by the decrease in the area of reacting surface with time and also possibly to a decreasing rate of penetration of the phosphate ions through the layer of lead phosphate. An attempt was made to test these points using a series of results obtained with the ultra-filter used by Giemsa[1] [1922]. It

[1] The rate of filtration with this type of ultra-filter was high using aqueous phosphate or Ringer solution.

was assumed that the velocity of chemical reaction at the surface was rapid compared with the rate of diffusion of phosphate ion. The calculated values of the velocity constant were found to fall rapidly with time. These values suggested that the process, instead of being governed throughout by the rate of diffusion, consisted of a reaction accompanied and eventually superseded by a much slower reaction, which was therefore presumably slow compared to the rate of diffusion of phosphate ion. The following facts support the view that the rapid reaction is a combination of phosphate and lead hydroxide and the slow reaction the combination of phosphate and lead carbonate.

(1) In the rapid reaction 90 % approximately of the combining phosphate disappears from solution. Approximately 10 % of the lead is actually present at the beginning as carbonate.

(2) It was found that the reaction proceeded at the same rate whether the mixture was vigorously stirred or maintained at rest. (The method could probably not detect any influence of stirring on the rapid reaction if such were present.)

(3) The very small solubility of lead carbonate in Ringer solution and serum (Jowett).

To obtain further information regarding the behaviour of lead carbonate with regard to phosphate ion the rate of combination of a coarse suspension of lead carbonate[1] with the phosphate ions of serum was measured. The results are given in Table VI and Fig. 2. The difficulty of obtaining a uniform sample of the suspension makes the absolute values in column 2 somewhat uncertain. The particles of the suspension used in (A) were smaller than those in (B); this probably accounts for the faster rate in the former case.

Table VI.

Room temperature.

(A) 50 cc. serum (0·00203 *M* phosphate).
3·0 cc. lead carbonate suspension = 1·2 % Pb.

Time (hours)	g. atoms P bound / g. atoms Pb	*k*
2	0·04	0·038
20	0·27	0·028
44	0·58	0·032

(B) 50 cc. serum (0·00203 *M* phosphate).
3·0 cc. lead carbonate suspension = 1·08 % Pb.

Time (hours)	g. atoms P bound / g. atoms Pb	*k*
7	0·08	0·021
12	0·11	0·017
30	0·30	0·020
77	0·65	0·021

In both cases excess lead carbonate was present which combined finally with all the phosphate present. In spite of the decrease in phosphate concentration the velocity remains practically constant. Assuming that the

[1] Prepared by mixing solutions of lead acetate and sodium bicarbonate both containing 0·5 % gelatin. The suspension settled almost completely in 60 minutes.

velocity is proportional only to the reacting surface (*i.e.* assuming that the chemical change is slow compared with the rate of arrival of phosphate ions at the surface) and that $PbHPO_4$ is formed, we would write

$$\frac{dx}{dt} = k\,(a-x)^{\frac{2}{3}},$$

a = initial g. mol. $PbCO_3$, x = g. mol. phosphate disappeared in time t, whence

$$k = \frac{1}{t}\,[a^{\frac{1}{3}} - (a-x)^{\frac{1}{3}}].$$

The calculated values of k are given in the third column, and are reasonably constant. The velocity of combination is also not inconsistent with the assumption that in the case of the experiments in Tables III–V the later slow disappearance of phosphate is due to the lead carbonate present in the lead suspension.

The lead ion content.

No detectable amount of ionic lead was found in the small volume of ultrafiltrates from the mixtures of serum (or Ringer solution) and the lead suspension. This would be expected, for however slowly the lead particles were converted into phosphate the lead ion concentration in the bulk of the serum would be governed by the solubility product of the phosphate and hence would be very small.

It may be noted that the values sometimes given for the so-called solubility of metallic lead in serum bear no relation to the resulting lead ion concentration. Such a solubility would depend chiefly on the amount of oxygen available for the oxidation of the lead to lead hydroxide and the peptising action of the serum on this compound or on lead phosphate formed therefrom.

The physical state of the lead particles in serum, etc.

The lead suspension was added to water, the phosphate solution, Ringer solution, and serum and the rate of settling at room temperature and at 37·5° observed. The rates were in the following order:

Ringer solution > water, serum > phosphate solution.

With the exception of Ringer solution at 37·5° (where precipitation occurs within 5 minutes) and to a lesser degree at room temperature, there is no marked acceleration of the slow rate of settling which takes place in the lead suspension itself.

The particles of the lead suspension are negatively charged. The increased stability in the phosphate solution may be due to an increase in this charge on conversion into lead phosphate and the decreased stability in Ringer solution may be due to the presence of the calcium ion. In serum there is the protective effect of the proteins to counterbalance any precipitating action of the various cations present. It may be noted in this connection that the addition of small amounts of lead acetate solution to serum gives a finely dispersed sol of lead phosphate [cf. Fairhall *et al.*, 1926, p. 24].

Conclusions.

It can be concluded from the results obtained that when the lead suspension is injected into the blood-stream the transformation of lead to lead hydroxide, and hydroxide to phosphate is rapid if there is rapid mixing with a sufficiently large volume of blood. In view of the speed of distribution of a dye-stuff throughout the blood (in about 5 minutes; cf. Lindhard [1926]) it is not improbable that a sufficient degree of mixing is attained to ensure the maximum velocity of chemical changes. Consequently the concentration of ionic lead in serum will fall almost immediately to that corresponding to the solubility of secondary lead phosphate. If, however, the particles temporarily reduced the phosphate concentration in the admixed serum to a very low value[1] the formation of lead carbonate would take place owing to the large amounts of bicarbonate ions present. Further mixing with more blood would again reduce the lead ion concentration to the value characteristic of lead phosphate.

Since, however, any given amount of a soluble lead salt under similar conditions would be transformed into lead phosphate (or carbonate) more rapidly than an equal amount of the lead suspension itself, it is necessary to explain the greater toxic effect of injection of the simple lead salt. It might be suggested that with the soluble salt a faster temporary local reduction of phosphate and bicarbonate concentration to a very low value would leave enough ionic lead in circulation for a sufficient time to cause injury. With the lead suspension the concentration of ionic lead could never be greater than that corresponding to lead hydroxide. An alternative and more probable view is that the fine sol of lead phosphate which would be formed from the soluble lead salt would be more toxic (greater number of smaller particles facilitating distribution, penetration, etc.) than the coarser suspension resulting from the introduction of the lead in the form of the lead suspension.

Summary.

1. The changes which take place when a colloidal lead suspension is added to an aqueous solution of sodium phosphate, to a Ringer solution containing sodium bicarbonate and phosphate, and to cow's serum have been investigated.

2. Ionic lead is not produced in any detectable amount on the addition of the lead suspension to these solutions. The phosphate ions unite with the particles which remain in suspension (except in the case of Ringer solution at 37·5°).

3. The final values obtained for the ratio g. atoms phosphorus bound/g. atoms lead present correspond with the formation of the tertiary phosphate $Pb_3(PO_4)_2$ in the aqueous phosphate solution. On the other hand, the secondary

[1] Assuming the plasma volume to be 1·515 l. [Lindhard, 1926] and an inorganic phosphorus content of 3·72 mg. per 100 cc. there is sufficient phosphate present to combine with 0·38 g. Pb as $PbHPO_4$. The maximum amount of lead suspension injected is 20 cc., *i.e.* 0·1 g.Pb.

phosphate $PbHPO_4$ is formed in Ringer solution and in serum. Using lead acetate in place of the suspension, the secondary phosphate is formed in serum and the tertiary salt in the other two cases. The formation of the secondary phosphate is attributed to delayed equilibrium.

4. The combination of phosphate ions with the particles of the lead suspension is preceded by the rapid oxidation of the metallic lead to lead hydroxide. The disappearance of the phosphate is due to two simultaneous reactions: (*a*) a rapid combination with the lead hydroxide, (*b*) a slow combination with the lead carbonate of the suspension. The results obtained with a suspension of lead carbonate alone show that the velocity of the latter reaction is not governed by the rate of diffusion of the phosphate ions to the reaction surface of the particles but by the slow chemical reaction at the surface.

In conclusion, the writer wishes to express his best thanks to Prof. W. C. M. Lewis, F.R.S., under whose direction this work was carried out.

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CIV. ANTINEURITIC YEAST CONCENTRATES.

II. THE USE OF NORITE CHARCOAL IN THE CONCENTRATION OF TORULIN.

By HENRY WULFF KINNERSLEY
and RUDOLPH ALBERT PETERS.

From the Rockefeller Department of Biochemistry, Oxford.

(*Received June 2nd, 1927.*)

In this paper are described improved methods of concentrating torulin to an activity of 0·5–1·0 mg. per day and some of the experimental evidence upon which the better methods are based. *Torulin* is here defined as the factor in yeast curative of symptoms in pigeons of head retraction induced by feeding upon polished rice. The work forms a continuation of that previously published [Kinnersley and Peters, 1925]. Discussion of the relation of this work to that of others is postponed to the end of this paper.

Experimental.

The methods of testing have been those previously described, and the activity (curative and protective) is given throughout in mg. per pigeon per day (day-dose). Previous methods of concentrating to an activity of 1·0 mg. involved a preparation of the Osborne-Wakeman aqueous extract from baker's yeast, and the successive removal of precipitates with neutral lead acetate and acid mercuric sulphate. At this stage in the earlier experiments, barium sulphide together with barium sulphate precipitated in the solution was used to clear metals and certain colloidal matter before charcoal treatment. Subsequently this was discarded owing to the tedium of filtration. Instead, the filtrate from the mercuric sulphate precipitate was brought to p_H 5·0 approx., any precipitate which appeared being removed. Charcoal was then stirred into the solution, and the antineuritic substance together with impurities removed from the charcoal by extraction with acid 50 % alcohol. The idea which guided these experiments was based upon the belief that the earliest possible treatment with charcoal shortened stages in which large volumes of solution must be handled. Working upon the small scale and with care in the use of the mercury reagent, satisfactory concentrates can be prepared in this way. The concentrates so obtained can be further purified by alcohol fractionation. Exp. 1 gives the details of one such experiment and exemplifies the technique.

Exp. 1. 7 lb. baker's yeast (D.C.L.) were extracted twice with tap water, the combined extracts making a volume of 2700 cc. and containing 100·9 g. organic solids. 280 cc. neutral lead acetate (25 %) were added, and to the filtrate sulphuric acid until acid to Congo red and then 70 cc. of mercuric sulphate reagent (Hopkins). The filtrate was brought to p_H 5·0 with NaOH (20 %), and a small precipitate removed by filtration. It was treated successively with 30 and then 10 g. norite charcoal, allowed to stand with stirring for about 10 minutes, and the charcoal collected upon a filter and well washed with distilled water. The combined charcoals were extracted with 200 cc. and 100 cc. 55 % alcohol by volume containing 1 cc. conc. HCl %. The organic solids present in the combined alcoholic extract were 2·850 g. The solutions were concentrated to about 5 cc. *in vacuo* at about 60°, and 45 cc. 97 % alcohol were added, making an alcohol concentration of 87 %[1]. The precipitate was removed by the centrifuge and washed with 87 % alcohol. The further treatment is sketched below:

Centrifugate — 87 % alcohol precipitate
Test 500 + 700, 700 doses
Alcohol removed, 50 cc. water added, and lead acetate (25 %) until no further precipate

Centrifugate. Passed H_2S 2 hours and washed precipitate with water containing trace of acetic acid — PbAc precipitate

Centrifugate. Boiled off H_2S and concentrated *in vacuo* to 4 cc.; added 95 cc. alcohol — Sulphide precipitate

Centrifugate
Test 600 doses
Concentrated with 1 cc. conc. HCl (to remove acetic) to dark brown gum. Extracted with hot 97 % alcohol. Washed the precipitate with 97 % alcohol — 95 % alcohol precipitate

Centrifugate. Removed alcohol and added NaOH (20 %) to p_H 5·0. To the volume 10 cc. added 90 cc. 97 % alcohol — 97 % alcohol precipitate

Centrifugate. Concentrated to about 2 cc. and added 48 cc. 97 % alcohol. The precipitate was redissolved in 3 cc. H_2O and reprecipitated with 47 cc. 97 % alcohol — 88 % alcohol precipitate

Centrifugate — 92 % alcohol precipitate
Test 330 doses
Concentrated nearly to dryness, added 45 cc. absolute[2] alcohol, washed the precipitate with 97 % alcohol

Centrifugate. Concentrated to dark gum, extracted with hot absolute alcohol, the precipitate being washed with absolute alcohol — 97 % alcohol precipitate

Centrifugate. Taken to dryness, and extracted with 50 cc. ho absolute alcohol — 98/99 % alcohol precipitate
Test 700 doses. Organic solids 0·966 g.

This experiment is chosen out of many, because though the yield was not so good as in others or the final activity quite 1 mg., it illustrates certain

[1] By volume. Concentrations of alcohol are to be taken throughout as concentration by volume.

[2] No precautions to dry the absolute alcohol have been taken.

points, in particular the best method of fractionating with alcohol. It appears to represent the simplest possible method of reaching this activity from the charcoal concentrates previously described. The principle involved is the removal of some interfering substance with lead acetate, removal of metals in acetic acid solution, followed by gradual removal of acetic acid and material insoluble in alcohol in acid solution. This is followed by a repetition of the same steps in rather more alkaline solution. The steps are fairly expeditious. Experience has convinced us that it is dangerous to venture from the path described. For instance, too rapid use of alcohol does not take to pieces the adsorption complexes efficiently, and there is much loss of activity upon the alcohol precipitates. Omission of the HCl after treatment with H_2S leads to the throwing out of torulin upon the alcohol precipitates. Improper removal of alcohol before bringing to p_H 5·0 is apt to cause destruction when soda is used, etc. It is abundantly clear that torulin of this activity is soluble in absolute ethyl alcohol containing mere traces of water.

The method exemplified in Exp. 1 can be relied upon to give some 700–800 doses from 7 lb. D.C.L. baker's yeast of an activity of about 5·0 mg. a day at the charcoal stage and about 1 mg. per day after alcohol fractionation. A trial of brewer's yeast did not give more activity, and the material was much more coloured.

When these methods were applied to large scale batches of yeast (56 lb.), it became evident that other factors intervened, complicating the subsequent fractionation. These and the slow filtration of the earlier stages led to a return to a more careful study of the pre-charcoal steps. It was found that by the removal of gum and subsequent adsorption of the torulin upon norite charcoal at p_H 7·0, the yield of active substance may be doubled, and the activity at the charcoal stage improved to between 0·5–1·5 mg. per day. Moreover, the speed of preparation is so much increased that it is possible for two persons to work up 14–28 lb. yeast in 2–3 days to give a yield of 3000–6000 day-doses of this activity.

It is proposed to present certain of the experimental evidence bearing upon the method of extraction used.

(1) *The use of baryta to clear gum and other colloidal matter.*

It has been found that treatment with cold saturated baryta after removal of the neutral lead acetate precipitates under certain circumstances rapidly flocculates the gum and clears the solution. Immediately following this step, sufficient sulphuric acid can be added to make acid and throw down the barium as sulphate which rapidly settles. This treatment much accelerates filtration in the subsequent stages. Certainty as to the exact mechanism of clearance has not yet been reached. It appears to be essentially a colloidal flocculation. It is suggested as an explanation that in the presence of traces of lead, baryta removes the gum as a coarse flocculent precipitate which in its turn adsorbs other colloidal substances. It is certainly not simple because the optimum

conditions vary slightly according to the condition of the yeast. Provided that the amount of lead is kept low, the losses at this stage are small. This is not so if the solution is cleared by the addition of baryta and basic lead acetate. In one case in which this was tried, a magnificent clearance was effected, but at the expense of large losses of active material by adsorption upon the precipitate. Table I gives some results.

Table I.

Exp.	Condition of material	Dose given	Bird test (days)	Total doses
2	Pb filtrate before baryta	1/3000	4·0	12,000
	Same after baryta	1/2200	5·0	11,000
3	Aqueous yeast extract before lead acetate	1/1000	3·5	3,500
	Same after lead and baryta stage	1/1000	6·0	6,000

In Exp. 3 there is an apparent increase in activity. As this has been noticed more than once it is probably not due to experimental error and is analogous to the increase in growth-promoting activity for yeast observed by Funk and Dubin [1920] after treatment of yeast extracts with lead acetate and mercury. It suggests that any estimates of actual torulin content obtained by the feeding of a natural food-stuff are apt to be erroneous. The inhibitory effect of impurities may be due either to an actual toxic antagonism or merely to a failure of absorption in the gut.

(2) *Specificity of adsorption by charcoal.*

Charcoal as an adsorbent for the antineuritic factor from rice polishings was first employed by Chamberlain and Vedder [1911, 1912]. These authors were not able to recover the active principle from the charcoal by extraction with ether, absolute alcohol or water. Cooper [1913] confirmed the extraction by animal charcoal from horse-flesh extract, but stated that water and alcohol would recover the factor. Eddy, Heft, Stevenson and Johnson [1921] used charcoal (norite) for the adsorption from alfalfa extract of the growth-promoting substance for yeast, part of which they attributed to vitamin B. They recovered the principle with glacial acetic acid. Funk and Dubin [1921] have employed norite for fractionating vitamin B and the yeast growth-promoting factor. Mueller [1922] used it for separating streptococcal growth-factors.

Earlier in this work the active principle was recovered from norite charcoal by the use of 50 % alcohol by volume containing 1 cc. HCl %. More lately this treatment has been varied, and also the norite charcoal itself purified as described by Merrill [1921] by boiling with strong HCl, washing until free from chlorides and drying. Such purified norite does not seem to be changed in adsorptive properties so far as this work is concerned. In the attempt to improve the use of norite for concentrating torulin, it became of importance to settle how far adsorption upon charcoal was a property of torulin itself. After treatment with mercuric sulphate, adsorption at p_H 5·0–7·0 is a property of the torulin in solution. Exp. 4 shows that it is not necessarily a property of torulin at a more active stage, viz. 1·0 mg. day-dose.

Exp. 4. Preparation from baker's yeast, made by the older technique and worked up to solubility in 99 % alcohol. Organic solids 0·525 g. Bird tests: 7 Aug. 1/100, 5 days; 26 Oct. 1/100, 4 days; activity 1·2 mg. Alcohol removed *in vacuo*, taken up in 50 cc. H_2O and brought to p_H 5·0 with NaOH, a small insoluble precipitate being removed by the centrifuge. Treated with 1 g. of washed norite charcoal, which largely decolorised the preparation. Filtrate from charcoal gave organic solids 0·425 g. Bird tests: 1/50, 8 days; 8 days, or 400 doses; activity 1·05 mg.

Hence at p_H 5·5 at a later stage charcoal does not extract torulin from solution when 10 doses to the cc. are present, although at an earlier stage it extracts it when not more than one dose is present to 1 cc. It is still possible to obtain some adsorption of torulin at p_H 7·0 and thereby a concentration of the active principle from a solution of activity 1·0 mg., provided that the torulin concentration is 50–200 doses per 10 cc., but a second treatment of such a solution adsorbs no more torulin, so that even here torulin is not itself being adsorbed.

It may be concluded therefore that adsorption of torulin by norite charcoal is not a property of torulin itself, but that it depends upon the presence of some co-adsorbent. The problem in the early stages therefore narrows down to the issue of removing the maximum of interfering substances without removing the co-adsorbent.

(3) *The optimum conditions for adsorption.*

This conclusion leads to the following three questions. What is the optimum p_H for adsorption? Does the optimum p_H differ for material that has undergone previous treatment with lead and baryta only, as distinct from that which has also been treated with mercuric sulphate? Is treatment with mercuric sulphate necessary? Exps. 5–9 were designed to settle these questions, all except the last being performed upon an extract from 56 lb. of baker's yeast, kindly made by Messrs The British Drug Houses, Ltd., so that the basic extract should be homogeneous. In Exp. 5 adsorptions at various p_H were made after mercuric sulphate treatment. In Exp. 6 the effect of a re-extraction at p_H 5·0 after one at p_H 1·0 was tried. In Exp. 7 adsorption at various p_H in absence of mercuric sulphate was tried, this being reinvestigated in Exp. 8.

Exp. 5. Preliminary treatment—lead acetate, baryta, and mercuric sulphate. The total filtrate from these precipitates, equivalent to some 2800 day-doses, was divided into four equal portions, which were brought by addition of NaOH to p_H about 1·0, 4·0, 6·0 and 7·0. Each portion was then treated with 15 g. followed by 5 g. of washed norite, each lot of charcoal being then treated with acid 50 % alcohol. Table II shows the tests for the 15 g. charcoal extracts.

Second charcoal extracts in the case of p_H 6·0 and 7·0 yielded 180 and 72 doses of activity 3·4 mg. and 9 mg.

Table II.

Exp.	p_H	Organic solids (g.)	Pigeon tests (doses)	Activity (mg.)	% extraction
5	1·0 approx.	1·248	186	6·7	27
	4·0	0·990	164, 82 } 120	8·2	17
	6·0	1·064	456, 760 } 603	1·8	86
	7·0	1·275	850, 680 } 760	1·7	108
6	5·0 (after 1·0)	0·976	120, 270 } 200	4·8	—

The most efficient extraction was at p_H 7·0, where in this case within the limits of error practically all the torulin was removed by one charcoal treatment, to yield material of an activity below 2 mg. The result at p_H 6·0 is near to this. The experiment shows how profoundly the charcoal adsorption is influenced by p_H.

We may next consider the effect of treating the filtrate from p_H 1·0 with fresh charcoal at p_H 5·0. This is seen in Exp. 6 (Table II). Assuming that some 100 doses were extracted by the second charcoal treatment at p_H 1·0 (this test was accidentally omitted), which is certainly an outside figure, there should be at least 414 doses remaining. Of these not more than half can be extracted at p_H 5·0 following the first charcoal treatments. It will be noticed that irrespective of p_H about 1·0 g. of organic solids is adsorbed by 15 g. of charcoal. After adsorption 48 g. of organic solids remained in the filtrate.

It may be asked now what is the effect of omitting mercuric sulphate, a step that is undesirable from several points of view. Exp. 7 (Table III) deals with this point.

Exp. 7. A volume of solution which had not been treated with mercuric sulphate, equivalent to some 2100 doses, was divided into three portions and treated as before with 15 g. washed norite charcoal, after being brought respectively to p_H 5·0, 6·0 and 7·0 with NaOH.

Table III. (*Exp.* 7.)

p_H	Organic solids (g.)	Pigeon test (doses)	Activity (mg.)	% extraction
5·0	1·300		3·4	55
6·0	1·036	200, 400 } 300 av.	3·4	43
7·0	1·170	304	3·9	43

In this experiment adsorption has been relatively inefficient, The presence of the substances in solution precipitable by mercuric sulphate has therefore depressed the adsorption at p_H 6·0 and 7·0 as compared with Exp. 5. This result was curious but has received later confirmation. In Exp. 8 the remainder of the batch was treated at p_H 6·0 and 7·0.

Exp. 8. The remaining material from the last experiments, as in Exp. 7, was treated at p_H 6·0 and 7·0 with 60 and then 20 g. of washed norite charcoal.

The method of extraction was varied in this case. Successive extractions with $N/10$ HCl, and then 50 % acid alcohol were employed for the first charcoal, and with 50 % acid alcohol alone for the second. Table IV gives the results.

Table IV. (*Exp.* 8.)

p_H	Charcoal (g.)	Extract by	Organic solids (g.)	Doses		Activity (mg.)	% extraction
6·0	60	$N/10$ HCl	1·127	840	1589	1·35	—
		50 % alc.	1·926	749		2·6	50
	20	50 % alc.	1·430	440		3·2	14
7·0	60	$N/10$ HCl	1·044	725	1165	1·44	—
		50 % alc.	2·354	440		5·4	38
	20	50 % alc.	1·44	?840*		—	—

* Probably an irregular bird.

Exp. 9 is a further variation of the same type. The extract from 14 lb. baker's yeast after treatment with lead acetate and baryta, and removal of baryta with sulphuric acid yielded a filtrate of 10,000 cc., which was divided into two equal portions *A* and *B*. To *A* 50 cc. mercuric sulphate reagent were added and the whole filtered immediately. To the filtrate from *A* and to *B* (untreated with mercury), sufficient NaOH was added to bring the p_H to 7·0. Each was then treated successively with 30 and 10 g. washed norite. Subsequently the charcoals were extracted with $N/10$ HCl and 50 % acid alcohol. Table V gives the results for the first charcoal (30 g.) extracts.

Table V. (*Exp.* 9.)

	Extract by	Organic solids (g.)	Doses	Activity (mg.)
A.	$N/10$ HCl	0·440	900 + 900+	0·49
	50 % alc.	1·400	1800+	0·78
B.	$N/10$ HCl	0·560	600+	0·93
	50 % alc.	1·592	Nil + 240	6·6 ?

Exps. 8 and 9 seem to confirm the fact that treatment with mercuric sulphate facilitates adsorption of torulin. It is possible to remove the substance precipitated by mercury in large part by treatment with charcoal at p_H 2·0, and to follow this with the usual adsorption at p_H 7·0. Experiments upon the yield of material show that it is as good for the $N/10$ HCl extract both as regards amount and activity, but the yield extracted by 50 % acid alcohol is not so good as following the mercury treatment. A detailed account of this method of treatment is given later, as it is likely to prove of value to those who require these concentrates for feeding experiments, owing to the complete freedom from mercury. Enough evidence has been adduced to show how variable is the character of an adsorption of this kind, and how dangerous it might be to draw conclusions about the adsorption of torulin from one class of mixture such as yeast extract and apply this to another. Such experience may be regarded as familiar to the enzyme investigator, as shown by the work of Willstätter and his colleagues upon invertase.

The experiments in this section permit us to draw the following conclusions in answer to the questions. The optimum p_H varies according to the mixture from which adsorption takes place. For material not treated with mercuric sulphate the optimum is p_H 5·0–6·0, and this adsorption is less efficient than adsorption at p_H 7·0 after treatment with mercuric sulphate. The mercuric sulphate stage or its equivalent cannot be omitted if the maximum yield is to be obtained.

(4) *The optimum conditions for extraction.*

It will have been observed in Exps. 8 and 9 that two extractions of the charcoal were made, namely, with *N*/10 HCl followed by 50 % acid alcohol. Further, the material extracted by *N*/10 HCl in this way tends to have a high activity, 0·4 mg. having been observed at this stage. In Exp. 9 the activity was 0·5 mg. This was rather unexpected in view of earlier experiments and suggested that the gum had modified conditions in previous work. It further appeared possible that the use of stronger alcohol as a first step might produce a differential extraction, and give even higher activity in the extract. Exp. 10 illustrates the effect of extracting first with strong alcohol.

Exp. 10. 14 lb. baker's yeast. Extraction of the charcoal was carried out first with 300 cc. 97 % alcohol containing 1 cc. conc. HCl %; allowing for water retained by the charcoal this means extraction with 85–90 % alcohol by volume. Subsequent extractions were with *N*/10 HCl and 60 % acid alcohol. Table VI gives the results obtained for the first and second charcoals.

Table VI.

Extract by	Organic solids (g.)	Doses	Activity (mg.)
1st charcoal:			
acid 97 % alcohol	2·450	1250} 1250}	2·0
N/10 HCl	0·560	500} 150}	1·7
acid 60 % alcohol	1·080	300} 300}	3·5
2nd charcoal:			
acid 97 % alcohol	0·852	360} 360}	2·5
N/10 HCl	0·130	100} 200}	0·9
acid 60 % alcohol	0·231	under 50	? 4·5

Total doses 1875 + 560 = 2435.

The material obtained in this way is not so good as that from the initial *N*/10 extraction. Nothing is therefore to be gained by the insertion of the strong alcohol step. It is clearly ineffective as a means of removing impurities, owing to the large number of doses extracted by the strong alcohol.

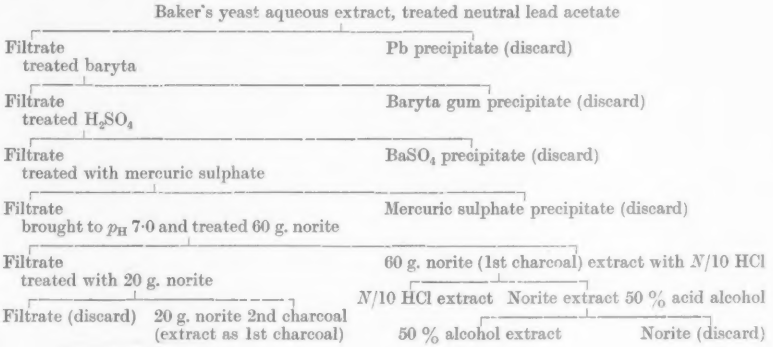
It may be further enquired whether anything is to be gained by an extraction with stronger alcohol following 50 %. This was tried in one case giving no appreciable yield of torulin although organic substances were still

extracted from the charcoal. In view of the above results, as a routine, two extractions of the charcoal are now made, one with $N/10$ HCl to which sufficient HCl is added to make the p_H definitely acid to Congo red if necessary. A further extraction is then made with 50 % acid alcohol by volume.

Optimum amount of charcoal. Experiment has shown that for the aqueous extract from 14 lb. of yeast, treatment with 60 g. of charcoal followed by a subsequent 20 g. cannot be improved.

Improved technique for concentrating torulin.

Based upon the foregoing experiments, the following technique has been elaborated.



Preparation of norite concentrate.

The description applies to 14 lb. of baker's yeast (D.C.L.). The yeast is allowed to remain 3 days at laboratory temperature, 15–20°, in the 7 lb. bags. Less yield seems to be obtained if this preliminary autolysis is omitted. An aqueous extract is then made by stirring the yeast gradually in small quantities into 3500 cc. of boiling tap water until all the yeast is added. (We cannot find any difference in results if acetic acid be added.) The mixture is not allowed to boil for more than 5 minutes. It is then filtered upon Büchner funnels with constant renewal of papers. This process takes about 2 hours. A second extraction with 2800 cc. of water is made. The whole operation of extraction takes about 4 hours and gives a volume of 7000 cc. The combined filtrates are treated with about 400 cc. of 25 % neutral lead acetate solution. This amount is more than the amount necessary to bring down the main bulky precipitate, but is short of complete precipitation. It seems to be the optimum for the next stage. The lead precipitate is removed by filtration overnight through folded filter papers in ordinary funnels. The speed of this filtration varies, most preparations filtering in a few hours. No attempt is made to wash the precipitates. The filtrate often clouds upon standing, but

need not be filtered again on this account. The filtrate from the lead precipitate measuring some 5500 cc. is treated with cold saturated baryta solution containing a little precipitated baryta in suspension. About 3000–4000 cc. are usually required. Immediate flocculation of the gum should take place which should then settle rapidly to the bottom of the vessel, clearing the solution to leave a crystal clear lemon yellow supernatant fluid.

It is well to test this step upon a small scale (50–100 cc.). If flocculation is not obtained by the addition of baryta, a small amount of the lead acetate solution, 2 cc. per litre, can be added, *before* adding the baryta, which will usually settle matters (see Section (1) of this paper). After flocculation of the gum, filtration is rapidly effected through folded papers, and the filtrate treated as soon as possible with sufficient sulphuric acid to make acid to Congo red. The barium sulphate, etc., are removed by decantation and filtration. At this stage the volume of the solution may measure about 10 litres.

Occasionally we have met with a yeast extract which will not filter overnight at the lead acetate stage. If this be so, the only course is to treat directly with baryta in the presence of the lead precipitate. The yield from such a preparation is not so good, the precipitates adsorbing torulin.

The filtrate from the barium sulphate, which need not be absolutely clear, is brought to p_H 2·5 (approx.) with addition of either sulphuric acid or of NaOH, and is then treated with mercuric sulphate in sulphuric acid, 80–100 cc. being used. It is then filtered through folded papers without delay. After filtration a faint cloud often appears in the solution upon standing, which is not removed. Care must be taken not to have excess of mercury present at this stage. The point to which we work is reached when 10 cc. of the solution taken into a test-tube ceases to give an immediate flocculent precipitate with addition of one drop of the reagent. At this point it gives a cloud.

After removal of the mercury precipitate, NaOH is added to p_H 7·0 (at which point the faint cloud if present redissolves) and the solution is treated with 60 g. dry purified norite charcoal. The charcoal is allowed to stand in contact with the solution with stirring for about 10 minutes, and is then collected upon a Büchner funnel. Filtration is usually rapid at this stage. The filtrate from the first charcoal is treated with 20 g. more of charcoal, this second charcoal being kept separate from the first. The charcoal precipitates are thoroughly washed with distilled water upon the funnel. The first and second charcoals are each treated separately as follows. Two extractions are made with $N/10$ HCl, *ca.* 200 cc., upon the hot water-bath, the papers and charcoal being placed with the HCl for this purpose in a beaker. If the extracting solution during the boiling becomes alkaline to Congo red, strong HCl is added drop by drop until the whole is acid to Congo red paper. After heating for 1 hour, the charcoal is filtered off while hot. A second extraction of the charcoal is then made in the same manner. These two almost colourless extracts are combined, dilute $BaCl_2$ is added to remove the last traces of sulphuric acid and the extracts are concentrated *in vacuo* at a temperature

not exceeding 60° to a volume of 50 cc. If a cold store is not available, sufficient alcohol is added to make a volume concentration of about 15 %. This acts as a preservative; in its absence we have found moulds growing in such solutions.

After extraction of the charcoal with $N/10$ HCl, a further yield of less active material can be obtained by extraction with 50 % alcohol (by volume) containing 1 cc. strong HCl %; two extractions are made, each with about 150 cc. acid alcohol.

The second charcoal extracts are worked up similarly; lately the extraction with 50 % acid alcohol has been omitted. The above operations take approximately 3 days, the last day being occupied in the extraction of the charcoals. We have been able upon occasion to work up twice the quantity in this time in the laboratory.

Yields. We have now worked up some nine 14 lb. preparations and eight 28 lb. preparations by variants of this technique, and have given above the one which we consider to be most satisfactory. The $N/10$ HCl extract for 14 lb. contains between 1200–2000 doses of activity 0·4–1·0 mg. The alcoholic extracts which are coloured give a yield of some 1500 more doses of activity 1·0–1·5 mg. The second charcoal $N/10$ HCl extract gives about 600 further doses of between 1·0 and 2·0 mg. activity. The 50 % extract of the second charcoal is relatively inactive.

The highest yield which we have obtained from 14 lb. yeast has been 4400 doses. This is only some 70 % of the total doses present in the gum-free aqueous extract, which has given us a figure corresponding to 6000 doses in the 14 lb. This latter figure corresponding to one dose in 1 g. of pressed yeast is much higher than we had originally supposed, and than the figure given by Cooper [1912] of 2·5 g.

Though the yields are doubled as compared with our previous methods, and are more active at the same stage, it appears that there is still some considerable loss in the processes connected with the charcoal stage. Tests upon the filtrate from the charcoal have not given sufficiently reliable results to enable us to draw conclusions as to the amount of torulin present after the charcoal adsorption.

The presence of traces of metals and of a relatively large amount of acid is an objectionable feature to those who wish to employ concentrates prepared in this way for prolonged feeding experiments. These can be eliminated by removal of alcohol *in vacuo*, bringing to p_H 4·5 and then treating with H_2S. After this, the fluid is warmed upon the water-bath until flocculation of any colloidal sulphide takes place, filtered, and then boiled over the naked flame until free from H_2S. It is then evaporated to a small volume after addition of a small amount of HCl to drive off any acetic acid, and then subjected to the alcohol fractionation as described in the introduction to this paper (Exp. 1), but leaving out the lead acetate stage. In cases in which this technique has been applied the material has concentrated to an activity of 0·15–0·3 mg. a day, giving material of N content varying from 15–25 %.

The following alternative technique is suggested for use in feeding experiments, charcoal being used instead of mercury.

Alternative technique avoiding the use of mercury.

After removal of the gum with baryta, the barium is removed with sulphuric acid so as to leave only a minimal excess of sulphuric acid in solution. HCl is then added to p_H about 2·5 and barium sulphate removed as usual. 60 g. of norite charcoal is then stirred into the solution. This removes the bulk of the substances precipitable with mercuric sulphate leaving the torulin in solution at this p_H. After filtration, the solution is brought to p_H 7·0, and the usual charcoal extractions made. Upon the four occasions when this technique has been followed there has been a good yield in the $N/10$ HCl extracts of a high activity 0·3–0·5 mg., but the extra amount obtained in the 50 % acid alcohol extracts is poor. The method has much to recommend it to those who do not wish to obtain the highest possible yield of active material, but are satisfied to get a reasonable yield with the least trouble.

Discussion.

It is hoped that the methods of obtaining reasonably active preparations of the curative substance with comparative ease may be of use to workers in many fields of nutrition. Now that such preparations can be obtained, there would seem to be no longer excuse for working with such impure mixtures as yeast autolysates as a source of this factor. It is not known yet what amount of torulin is needed in the nutrition of the rat, a matter which it is hoped to investigate shortly. This opens the necessity of discussing the place of torulin among the water-soluble vitamins, as also the excuse for the retention of this word. When this work was commenced, some years ago now, it was decided to adhere to the older curative test in spite of its many deficiencies because it seemed to be more likely to be testing for one factor than any test which involved the maintenance of weight or growth. Recent work upon the durability of vitamin B has tended to strengthen the view that this is so [see Harege and Carrick, 1926]. More especially Goldberger, Wheeler, Lillie and Rogers [1926] have shown that two factors are needed for the growth of rats, one of which is thermostable and present in autoclaved yeast, believed to be the pellagra-preventing factor, and both of which are of course present in fresh yeast. By the use of charcoal concentrates prepared by the early methods described by one of us [Peters, 1924], Chick and Roscoe [1927; Roscoe, 1927] have shown that the thermolabile factor for rat growth is present in the concentrates of the curative substance for pigeons (torulin concentrates).

It is therefore tempting to identify torulin with the antiberiberi vitamin, but this can at present be done only with reserve. It does not seem quite clear that symptoms of head retraction in the pigeon are certainly identical with beriberi [Eijkman, 1913], and it must be further remembered that

preparations of an activity of even 0·1 mg. per day for the pigeon are still sufficiently impure to admit of the presence of more than one factor. Some evidence that more than one factor may still be present has been obtained already in collaboration with Miss Reader, in work which is now proceeding. The various workers who have been recently directing their attention to the problem of isolating the antineuritic vitamin would appear to be investigating different problems. Levene and van de Hoeven [1926] using the growth of rats for their tests have obtained preparations of which 0·08 mg. daily are required for the rat. Seidell [1926] uses the maintenance of weight in pigeons as a standard test. This test has also been employed by Funk and Paton [1922]. Both of these tests would seem likely to test for one factor, only so long as all the others are supplied in the diet or from stores accumulated by the animal.

In view of the uncertainty and differences of opinion as to the tests to be employed for the so-called antineuritic factor, it is probably best for the present to use the term torulin, as defined in the introduction to this paper.

Quite recently Jansen and Donath [1926, 1927] have described the isolation of a crystalline substance from rice polishings, making use of a protective test in birds. The substance described is protective to *Munia maja* (a small tropical bird) in doses of 0·002 mg. a day, and to the pigeon in doses of 0·01 mg. per day. This would be eight times more active than the preparation previously described by the authors [Kinnersley and Peters, 1925]. No mention, however, is made as to whether this substance is also curative. In connection with preventive tests, it will be remembered that Funk [1913] showed that even synthetic allantoin and hydantoin would lead to diminished loss of weight by pigeons upon polished rice, and also tend to delay symptoms of polyneuritis.

The interesting results of the Dutch workers led us to apply their methods to some of the 0·4 mg. a day material obtained in the course of this work. We are obliged, however, to record our disappointment, having been unable to concentrate the torulin present by the use of their technique, starting in one case at the silver stage, and in another at the platinum stage. It would appear therefore that their methods are not applicable to the concentration of torulin in these fractions. Nor can we obtain evidence that the Pauly reaction fractionates with the activity, material of activity 0·1 mg. giving a much less intense Koessler and Hanke [1919] reaction than the less active material from which it had been derived, when equi-dose amounts were compared. An amount equivalent to two torulin doses of activity 0·1–0·2 mg. per day gave a yellow reaction of less colour intensity than the reddish reaction given by 0·01 mg. histidine hydrochloride.

We lay no stress upon our failure to concentrate torulin in our fractions by a method applicable to the protective (? antiberiberi factor) in rice polishings. Doubtless it lies in some peculiarity of the yeast extracts[1]. It is, however, possible that the antiberiberi factor is distinct from torulin. The question at

[1] Assuming that the curative substances in yeast and rice polishings are identical.

issue seems to be whether torulin is distinct from the antiberiberi factor, and what relation these properties of extracts bear to the protective factor in the sense of Jansen and Donath. It is indeed possible that the train of symptoms cured by torulin can only arise when absence of some other factor has accentuated a special phase of metabolism. This would explain certain abnormal responses to test doses, and would also justify the feeling that curative tests upon pigeons are not a reliable guide where human beriberi is concerned [Eijkman, 1913; Jansen and Donath, 1927].

SUMMARY.

Improvements in methods of concentrating torulin are described, torulin being defined as the factor in yeast curative of symptoms of head retraction in pigeons induced by a diet of polished rice. By a removal of gum with baryta, subsequent procedures are simplified. Adsorption upon norite charcoal is not a property of torulin of activity 1·0 mg. per day, and is therefore due to a co-adsorbent in the earlier stages. The optimum p_H for adsorption upon norite charcoal is 7·0, provided that the extract has undergone a treatment with mercuric sulphate, but this is not true if this step be omitted. An improved technique for obtaining torulin concentrates of an activity of 0·2–1·0 mg. per day is described, based upon the above observations.

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CV. DIGESTION IN THE COCKROACH.

I. THE HYDROGEN ION CONCENTRATION IN THE ALIMENTARY CANAL.

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Although there is a considerable literature on the chemistry of digestion in insects this contains little more than records of the presence or absence of particular enzymes and there have been few attempts to investigate the properties of such enzymes more closely. The present work has been undertaken as a preliminary to the study of digestion in blood-sucking and disease-carrying insects; for it seemed desirable before dealing with such specialised material to make a more or less detailed study of the digestive enzymes in some insect of generalised structure and omnivorous habits, to compare the properties of these enzymes with those from other sources which have been the subject of detailed examination in the past and to relate these properties with the conditions under which digestion is normally carried on. It was hoped that such an investigation would serve as a useful point of reference in subsequent work, while at the same time the experiments have afforded an opportunity for the development of a technique for obtaining quantitative results with very small amounts of material.

The insect selected for this purpose was the small German Cockroach (*Blatella germanica*), but in most cases the experiments have been repeated and confirmed on the large American Cockroach (*Periplaneta americana*).

The present paper deals with the reaction of the intestinal contents of this insect and the factors by which this reaction is influenced.

The reaction of the intestinal contents of the cockroach.

From the small amount of work which has been done on the reaction of the intestinal contents of insects [Crozier, 1924; Bodine, 1925; Senior-White, 1926], it appears that they do not as a rule show the extremes of acidity and alkalinity which are found in higher animals, though Jameson and Atkins [1921] record a p_H of 9·2 in the mid-gut of the larva of the silkworm.

The reaction of the crop contents of the cockroach has been variously stated to be weakly acid or neutral, the acidity when noted being usually attributed to changes in the food during digestion [Plateau, 1873]. Bodine

[1925], using the method of Felton [1921], measured the p_H of the gut contents of various grasshoppers (which belong to the same natural Order as the cockroach) and gives the p_H of the crop as 5·0–5·9 and of the mid-gut as 5·75–6·9, becoming less acid towards the hinder end. The p_H of the hind-gut and rectum varies from 6·4–7·2.

Methods.

The fluid available for the estimation of p_H in the gut of *B. germanica* amounts only to a few cubic millimetres. Approximate results may be obtained on this material by the rough method of Jameson and Atkins [1921]. But by the use of waxed plates and pipettes and the consequent elimination of the capillarity of the fluids a more exact colorimetric method has been developed.

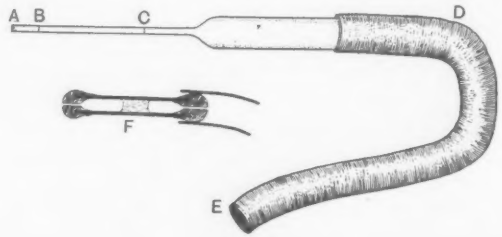

Fig. 1.

A capillary pipette (Fig. 1) is lined with a thin film of paraffin wax and two arbitrary graduations made at *B* and *C*, the volume *A–B* being about 2 mm.^{3}. The pipette is filled from *A–B* with the fluid to be tested, and then a solution of an appropriate indicator suitably diluted with boiled distilled water is run in until the fluid reaches *C*, the volume *B–C* being about 10 mm.^{3}. The accurate manipulation of these minute quantities of fluid requires a little practice, but is most readily effected as follows. A piece of rubber tubing is attached to the end of the pipette. The end *E* of this tubing is held in the teeth and the point *D* between the finger and thumb. Fluid is drawn in by compressing *D*, closing the teeth on *E*, relaxing *D* until the required volume has been sucked up and then releasing *E*. Fluid is expelled by the reverse process of closing *E*, compressing *D* and releasing *E* at the required moment. It is of course of the utmost importance *never to expel the fluid by blowing*. If the pipette has been well lined with wax and the fluid is expelled slowly and evenly the latter will be transferred quantitatively from the pipette. The fluid should be mixed by drawing it in and out several times.

The p_H of the fluid is determined by comparing it with mixtures made up with the same indicator solution in the same pipette, using standard buffer solutions in place of the test fluid in *A–B*. The colour comparison may be effected by expelling the drops of fluid on a white tile covered by a film of wax, where they form little spherical globules. But the evaporation of such drops is rapid and their exposure to the air is undesirable. A more satisfactory

method of comparison is the following. A length of hard glass tubing of about 1·5 mm. bore is tested by means of a short column of mercury and those pieces of uniform bore are selected. It is then well washed with distilled water and tested for alkali by standing in a dilute solution of phenol red for 12 hours [Martin and Lepper, 1926]. Finally, the selected portions are dried, cut into lengths of about 1 inch and their ends sealed by dipping them in melted paraffin wax (*F*, Fig. 1). When required for use the ends are perforated with a fine needle. They are filled with the series of standard buffer mixtures or with the test mixtures by means of a rubber tube attached to one end, and the ends are then sealed by touching them with a heated glass rod. The closure of the ends of the tubes with paraffin caps has the twofold advantage of allowing them to be readily sealed and of enabling one to take up the fluid quantitatively from the waxed tile. Using the volumes indicated in the above description the column of fluid to be observed is about 5 mm. long.

With practice the whole procedure from the dissection of the insect to the colour comparison can be effected in half a minute. The standard buffers employed were of the Clark and Lubs series; colour differences of 0·1 p_H can be detected readily. The indicators used were the phenol red, bromothymol blue, bromocresol purple, methyl red and bromophenol blue of Clark and Lubs. The standards were made up fresh each day. The waxed tiles and other apparatus require very careful washing with distilled water between each experiment.

The method is a dilution method and as such is applicable only when the solution to be tested is adequately buffered. That this is the case with the saliva and gut contents of the cockroach was demonstrated as follows. A series of cockroaches fed upon different diets was dissected and the crop contents made up with suitable indicators as above described; but from each specimen several samples were taken. In one, the volume *A–B* would be filled with the test fluid; in another, it would be partly filled and made up to *B* with distilled water. In this way dilutions varying from 1:6 to 1:12 were compared. In the great majority of cases the results obtained were identical and in few cases did they differ by more than about 0·1 p_H unit.

A rough check on the results has been obtained by the method of Jameson and Atkins [1921] and, where the amount of fluid available has permitted, the results have been further controlled by using more than one indicator.

The dissection of the cockroach is very easily accomplished. The insect is anaesthetised with chloroform, laid on its back, and, while the body is steadied between the finger and thumb, the head is seized with forceps and gently drawn forwards. Almost invariably the salivary glands with the salivary receptacles distended with fluid and the entire gut with its contents as far back as the malpighian vessels may be drawn through the neck of the insect and placed upon the waxed plate. The various regions of the gut are then punctured and the escaping fluid rapidly taken up into the pipette.

Results.

Table I shows the results obtained from a series of freshly caught cockroaches which had been feeding on a varied mixed diet. The "saliva" in each case was the clear fluid distending the salivary receptacles.

Table I.

Region of gut	No. of estimations	Range of p_H	Average p_H
Saliva	22	6·7–7·4	6·9
Crop	12	4·6–6·0	5·2
Mid-gut	9	6·0–6·4	6·2

These results are in fair agreement with those obtained by Bodine on grasshoppers. There is a definite production of acid in the crop.

It was of interest to determine the source and nature of this acid. The digestion of the cockroach takes place mainly in the crop, the digestive juices coming from the salivary glands and the mid-gut. The reaction in both these regions is less acid than in the crop and this suggests that the acid is produced in the crop itself. The degree of acidity is too slight to be due to a strong acid such as hydrochloric and Gunzberg's test proved negative. Biedermann [1898], who noted a similar mild acidity in the mid-gut of mealworms, considered that this was due to an acid salt of phosphoric acid.

A series of *B. germanica* was fed on a mixture of 5 % gelatin and sucrose and dissected 5 hours later. The fluid from the crops of these insects was collected and showed a p_H of about 5·0. A portion of this fluid was mixed with appropriate quantities of Briggs' [1922] phosphate reagents in a capillary tube, but no trace of phosphorus could be demonstrated, although very small quantities can be detected by this method. On the other hand, the fluid gave a bright lemon yellow colour with dilute ferric chloride and there is little doubt that the acidity is due to lactic or other organic acids. It seemed probable that these acids are produced during digestion, and if such be the case the reaction should vary with the food taken. This point is the subject of the experiment given in Table II.

Table II.

Series	Food (mixed with water)	p_H of crop			p_H of mid-gut		
		No.	Range	Average	No.	Range	Average
A	Caseinogen	13	5·6–6·9	6·3	6	6·2–7·0	6·4
,,	Sucrose	10	4·4–5·6	4·8	7	6·2–6·4	6·3
B	Caseinogen	5	6·2–6·6	6·4	2	6·0–6·6	—
,,	Fibrin	2	5·8–6·4	—	1	6·2	—
,,	Gelatin	3	6·2–6·6	6·4	1	6·2	—
,,	Starch	3	5·2–5·8	5·5	—	—	—
,,	Maltose	2	5·6–5·8	—	1	6·4	—
,,	Lactose	2	5·6–6·0	—	—	—	—
,,	Sucrose	5	4·4–5·6	4·7	3	6·2–6·4	6·3
,,	Glucose	3	4·4–4·6	4·5	2	6·2	—

Series *A* starved for 2 days and dissected 3 to 18 hours after feeding. Series *B* given nothing but water for 20 days and dissected 4 to 6 hours after feeding.

It is clear from these figures that acid is being produced from carbohydrate and that this acid production is much more intense with sucrose and glucose than with the other carbohydrates. It will be shown in a subsequent communication that sterile extracts from the gut of the cockroach have no glycolytic action; it is therefore highly probable that the acidity is due to the activity of micro-organisms.

Microscopic examination of smears prepared from the crop contents of wild cockroaches showed a great variety of bacilli, cocci and small yeasts, and it was noted that yeasts were far more abundant in crops with a high acidity (p_H 4·6) than in those with a more nearly neutral reaction.

Finally, an emulsion of the crops of wild cockroaches was incubated with various sugars. The details of this experiment are given in Table III, and it will be seen that acid is produced most readily from glucose, less readily from maltose and least so from lactose. No gas was produced in any of the cultures. Stained films from the cultures showed the presence of yeasts, bacilli and streptococci.

Table III.

A. Equal quantities of an emulsion in distilled water of the crops of *B. germanica* incubated with 10 cc. of 2 % solutions of sugars and 10 drops of methyl red, under sterile conditions.
Controls (*B*) are the same using boiled emulsion.
Y = yellow to methyl red. The figures give the approximate p_H.

	Glucose		Maltose		Lactose	
Time	*A*	*B*	*A*	*B*	*A*	*B*
11.0 a.m.	Y	Y	Y	Y	Y	Y
3.0 p.m.	5·2	Y	Y	Y	Y	Y
4·0 p.m.	4·8	Y	5·8	Y	Y	Y
9·30 a.m.	4·4	Y	4·6	Y	4·8	Y

Discussion.

It is evident from the foregoing experiments that the acidity commonly observable in the crop of the cockroach is not a physiological constant for the animal but is dependent on bacterial action. None the less, since the cockroach feeds mainly on carbohydrates, a certain degree of acidity represents the normal reaction of that part of the gut where digestion mainly occurs; and no matter what the origin of this acid the digestive enzymes must be capable of acting over that range of p_H observed in the living animal. This subject will be considered in subsequent communications.

The variability of the reaction in the crop is in striking contrast with the relative constancy in the mid-gut. This is doubtless due to the neutralisation of the acid by the secretion of the mid-gut and perhaps also of the malpighian tubes. The reason for the failure of acid fermentation to continue in the mid-gut is not clear. Most probably it is due to the rapid absorption of sugar. For example, it was found that after a meal of sucrose the crop contents always reduced Fehling's solution strongly, whereas a much less intense reduction was obtained with the contents of the mid-gut. Nothing is known of the regulating mechanism of the sphincter of the proventriculus (gizzard) by which the passage of food from the crop to the stomach is controlled.

SUMMARY.

1. A colorimetric method for the determination of p_H in about 0·002 cc. of fluid is described. The essential feature of the method is the use of waxed plates and pipettes for the manipulation of the fluid.

2. In wild cockroaches (*Blatella germanica*) the average p_H of the saliva is 6·9, of the crop contents 5·2, and of the stomach 6·2.

3. The acidity of the crop is shown to be due to the action of micro-organisms, particularly yeasts, upon sugar. After protein food the average p_H is 6·3 in the crop and 6·4 in the stomach. After carbohydrate food these figures are 4·8 and 6·3 respectively.

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CVI. DIGESTION IN THE COCKROACH.

II. THE DIGESTION OF CARBOHYDRATES.

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The most recent account of the digestive processes of the cockroach is that given by Swingle [1925], who employed the Common Cockroach (*Blatta orientalis*) in his experiments and whose conclusions are briefly as follows. The salivary glands produce only an amylase. The hepatic caeca of the mid-gut produce an invertase, maltase, lipase and protease. The remainder of the mid-gut (stomach) secretes the same enzymes though less abundantly. The protease he divides into a "trypsin" and an "erepsin"; but the tests employed for erepsin were (1) the formaldehyde titration of a peptone digest, and (2) the production in this digest of nitrogenous compounds not precipitated by phosphotungstic acid; both of which tests would be given equally well by trypsin alone. The hind-gut does not secrete digestive enzymes. The greater part, if not the whole, of digestion takes place in the crop, the enzymes passing backwards from the salivary glands and forwards from the mid-gut. The crop is lined by a membrane of chitin and is generally regarded as playing no part in the secretion of digestive ferments; but Swingle claims that extracts of washed crops contain traces of invertase, maltase and lipase, and the same claim is made by Sandford [1918] for lipase. Swingle does not decide from his evidence whether these enzymes are formed in the cells of the crop or absorbed by them from the digestive cavity.

It may be mentioned here that the results of Swingle have been confirmed, but that, in addition, an invertase has been found in the salivary glands of the German Cockroach (*Blatella germanica*), though not in the American Cockroach (*Periplaneta americana*) and that small quantities of lactase have been demonstrated in extracts of the mid-gut. With regard to the enzymes of the crop, the results of Swingle have been repeated by his method. The reliability of these results depends, however, upon the successful washing of the crops, a procedure which is not easily carried out, since the crops contain earthy material which is often very difficult to remove; and it has been shown that if the gut is washed out thoroughly in the living animal by giving it water but no food for a fortnight or three weeks, extracts of the crop are free from maltase and invertase, while the other parts of the digestive system retain

their normal properties. These results conform to the more orthodox view that the crop plays only a passive rôle in digestion. The methods employed in these experiments will be described later when dealing with the enzymes in detail.

This paper will deal with those enzymes of the cockroach which are concerned in carbohydrate digestion, with a view to comparing them with the corresponding enzymes from other sources. It has been found [Wigglesworth, 1927] that these enzymes normally function in a medium rendered acid (p_H 5·0) by the action of micro-organisms. In the present communication it will be shown that they are adapted to work at this reaction; but that with this exception their properties are very similar to those of the enzymes with which they have been compared.

The most clear-cut distinctions between enzymes of a given kind are to be found in the effects of various ions upon their activity, and this part of the subject has received most attention here. The effects of substrate or enzyme concentration upon the kinetics of their action appear to be less important. Temperature effects are so dependent upon other conditions of the experiment that few clear comparative data are available, and this aspect of the matter has not been touched.

The comparisons which have been made are far from complete, but they are sufficient to indicate that the enzymes concerned in carbohydrate digestion in the cockroach are very similar to those of yeast and of higher organisms.

Amylase.

Amylases from various sources differ in their optimum p_H, in the effect of inorganic ions upon their amyloclastic and saccharogenic activity [Sherman and Tanberg, 1916] and in the ratio between these two measurements of activity under a given set of conditions [Sherman and Schlesinger, 1913]. The salivary amylase of man is characterised by being inactivated by dialysis, and by having its activity (both amyloclastic and saccharogenic) restored by the anions of salts in the following descending order: chlorides, bromides, nitrates, iodides, phosphates and sulphates. The optimum concentration of chloride is from 0·003 to 0·03 N [Willstätter, Waldschmidt-Leitz and Hesse, 1923]. The optimum p_H varies from 6·1 to 6·9, depending upon the salts present [Michaelis and Pechstein, 1914].

In the alimentary canal of the cockroach, amylase is confined to the salivary glands.

Methods.

Preparations of active enzymes. Stock preparations of enzymes from the cockroach have been obtained in two ways.

(i) Before dissection the insects are given nothing but distilled water for a week or a fortnight in order to clean out the alimentary canal. The gut is removed from the insect as previously described [Wigglesworth, 1927] and

divided up into salivary glands, crop and mid-gut. These parts are collected from about twenty *B. germanica* and ground up in a small glass mortar with a little washed sand and a drop of glycerol until a fine uniform emulsion is obtained. This is then made up to about 10 cc. with 50 % glycerol to which are added a few drops of 5 % thymol in alcohol. This will be referred to as a "glycerol extract."

(ii) Dissection is carried out as before but the tissues are ground up with sand and a drop of water and of 5 % thymol in alcohol. The resulting emulsion is dried at room temperature and powdered. This will be referred to as a "dry preparation."

Human ptyalin was obtained by holding in the mouth a mixture of equal parts of glycerol and water and filtering the mixture.

General method of experiment.

(i) *Amyloclastic experiments.* The reactions are carried out in small test-tubes (100 mm. by 6 mm.), the total volume of fluid present being 1 cc., of which 0·5 cc. consists of a 2 % solution of soluble starch. A small pipette graduated in divisions of 0·025 cc. is placed in each tube at the outset of the experiment. A water-bath at 37° has been used throughout. At suitable intervals 0·025 cc. is withdrawn and run into 0·5 cc. quantities of very dilute (almost colourless) acidified iodine solution measured out into a series of uniform test-tubes. When the optimum of a series of conditions is sought, the method of Michaelis [1922] is adopted, *i.e.* the series of reacting mixtures is tested at an equal interval from the outset of the experiment and the one showing the greatest degree of change taken to represent the optimum. When the relative activity over a range of conditions is sought, the time taken to reach a suitable mauve tint in the fastest tube is noted. This tint is then used as an arbitrary end-point and the time taken by each mixture to reach this colour is observed. The reciprocals of these respective times are taken to represent velocity of action and, expressed as percentages of the fastest rate, are reported in the various experiments as "relative velocity." An additional check on the results has been obtained by noting the time taken to reach the achromic point. But for general use the former method has proved more satisfactory because in the slowly reacting mixtures the achromic point may never be attained, while in the rapidly reacting mixtures there is nothing to indicate whether it has been reached some time past.

Where two enzyme preparations are compared under the same series of conditions, the concentration of each is so adjusted before the experiment that it shows approximately the same activity as the other when both are at their respective optima. It is most convenient if the arbitrary end-point is reached in the fastest tube in about 15 minutes.

(ii) *Saccharogenic experiments.* The experiments are carried out precisely as above except that pipettes graduated in 0·1 cc. divisions are employed and the sugar produced in a given time is estimated by the method of Hagedorn

and Jensen [1922][1]. The concentration of enzyme is adjusted to give about 0·25 % of "glucose" in 15 minutes in the fastest mixture. The blank on such mixtures is so small that no preliminary precipitation is necessary. The amount of phosphate present in the phosphate buffer mixtures has been shown to have no effect on the reducing power of sugars as estimated by this method [cf. Visscher, 1926]. In these experiments it is assumed that the velocity is directly proportional to the amount of reducing sugar (expressed as "glucose") which is produced in a given time. This expression assumes that the hydrolysis of the starch is a linear function of time, an assumption which is probably only approximately true and which is only justified because no far-reaching conclusions are based upon it.

Results.

(i) *The effect of salts.* An experiment showing the effect of salts on cockroach amylase is given in Table I.

Table I.

Mixtures in reaction-tubes made up as follows: 0·1 cc. glycerol extract of salivary gland of *B. germanica* + 0·1 cc. 0·5 *M* solution of salt + 0·3 cc. distilled water + 0·5 cc. 2 % soluble starch.

Salt	Relative activity (amyloclastic)
None added	30
Sodium sulphate	47
Sodium phosphate (p_H 6·8)	50
Sodium nitrate	74
Potassium iodide	80
Sodium bromide	100
Sodium chloride	100

This experiment shows that the amyloclastic power of cockroach amylase is influenced by salts in the same way as ptyalin. In an experiment where the saccharogenic activity was measured, this was found to be influenced in the same way by the addition of salts.

A dry preparation of cockroach salivary gland was extracted with distilled water for 24 hours and the extract dialysed in a small collodion sac, prepared by the method of Gates [1922], against several changes of distilled water for 24 hours. The 2 % starch solution was similarly dialysed. In the absence of added salt the preparation so treated was almost inactive. A comparison of amyloclastic activity at various concentrations of sodium chloride was then made. These results are given in Fig. 1 and show an effect identical with that described in the case of human ptyalin. It is of interest to note that the amount of enzyme present in each of these mixtures was equivalent to one-fiftieth of the salivary gland of the cockroach.

[1] Since Hagedorn and Jensen make no reference to the fact and as I have found so many workers unacquainted with it, I may mention here that the *N*/200 solution of sodium thiosulphate will remain perfectly stable in ordinary distilled water for many weeks if this be made just alkaline with sodium hydroxide. Thus the titre of a solution of thiosulphate in distilled water against standard potassium iodate changed from 18·0 to 22·4 in 17 days, whereas a similar solution made just alkaline with sodium hydroxide changed from 18·1 to 18·2 in 2 months.

(ii) *The effect of hydrogen ion concentration.* In considering the "adaptation" of an enzyme to its environment, the precise concentration of hydrogen ions necessary for its optimal activity is of less importance than its ability to function over that range of p_H which it is liable to encounter in its sphere of action. It is on this account that in the experiments here reported more attention has been paid to the relative activity of the enzymes over a wide range of p_H than to the determination of the absolute optimum. Moreover, differences in activity around the optimum are so slight that a more reliable basis of comparison is afforded by considering the p_H at which activity is reduced by 50 %, irrespective of any theoretical interpretations [Michaelis and Davidsohn, 1911] which may be put upon this figure.

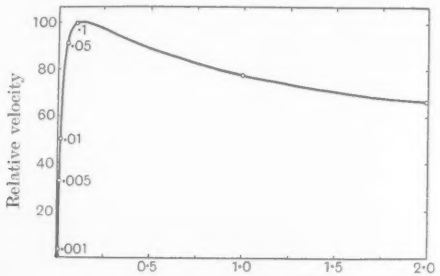

Fig. 1. Effect of sodium chloride on dialysed amylase from the salivary gland of the cockroach.

The glycerol extracts prepared as above described are practically unbuffered and the required hydrogen ion concentration has been obtained by taking in each tube 0·1 cc. of glycerol extract (after dilution if necessary), 0·1 cc. 1 % sodium chloride and 0·3 cc. of buffer. 0·5 cc. of 2 % soluble starch is added at the last moment. The phthalate and phosphate buffer mixtures of Clark and Lubs have been used. (Borate buffers cannot be employed in the presence of glycerol because of the production of the highly dissociated glyceroboric acid.) The p_H has been re-determined colorimetrically at the end of the experiment and shown to be unchanged.

Under these conditions the optimum p_H for cockroach amylase was found to be about 5·9 and in the case of human ptyalin about 6·5.

The relative activity over a wide range of p_H is shown in Fig. 2, which was obtained from a preparation from *Blatella germanica*. From this it will be seen that whereas the cockroach amylase still shows 50 % activity at p_H 4·8, human ptyalin is almost inactive at this reaction. The cockroach enzyme is much more tolerant of acid, a finding which was to be expected from the conditions obtaining in the gut of this insect [Wigglesworth, 1927]. The relative difference in activity in the alkaline direction is less striking. Twelve hours after the conclusion of this experiment digestion was still incomplete

in the more acid mixtures. The p_H in the various mixtures was brought back to about 6·4 with potassium hydroxide and acetic acid, and 0·5 cc. of 2 % starch added. In the tubes at p_H 4·0 both amylases had been destroyed. At less acid reactions the enzymes had been partially destroyed, that of the cockroach being the more resistant. Clearly the diminished activity in the acid region is due partly to enzyme destruction and partly to a reversible inactivation.

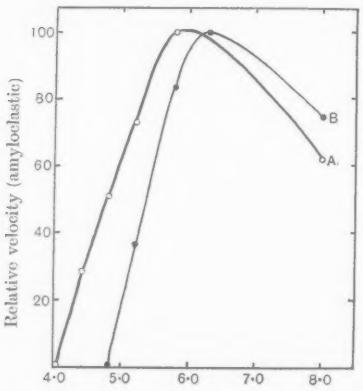

Fig. 2.

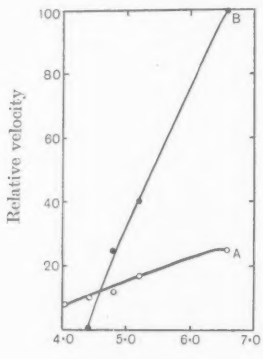

Fig. 3.

Fig. 2. p_H-activity curves (amyloclastic) for amylase. *A*, Salivary amylase of *Blatella germanica*. *B*, Human ptyalin.

Fig. 3. p_H-activity curves for amylase. *A*, Salivary amylase of *Periplaneta americana*. *B*, Human ptyalin.

Fig. 3 shows a similar experiment with an amylase preparation from the salivary gland of *Periplaneta americana*. Owing to an error in calculation the human preparation was nearly four times as active as the cockroach preparation at about the optimum; yet the activity of the cockroach amylase falls off far less rapidly in the acid direction.

Fig. 4 is a comparison of the two p_H-activity curves in an experiment where sugar production was taken as a measure of activity. This gives exactly the same results as the amyloclastic experiments.

It was of interest to determine whether the greater resistance of the cockroach amylase to acid was due to differences inherent in the enzyme or to some associated factor; for it is well known that the optimum p_H of amylases is markedly affected by the presence of salts [Michaelis and Pechstein, 1914]. This point was tested in the experiment given in Table II. Preparations of ptyalin (*A*) and of amylase from the German Cockroach (*B*) were adjusted to have the same activity at their p_H optima. Mixtures were made up as shown in the table and their activity compared at p_H 4·4. The "inactivated"

enzymes were left for 5 minutes in a boiling water-bath and were shown to be completely inactive.

Table II.

Mixture	Relative activity
0·1 cc. B + 0·1 cc. "inactivated" A + 0·1 cc. 1 % NaCl + 0·2 cc. phthalate buffer p_H 4·4 + 0·5 cc. 2 % starch	100
0·1 cc. B + 0·1 cc. 50 % glycerol: the rest as before	100
0·1 cc. A + 0·1 cc. "inactivated" B: the rest as before	12·5
0·1 cc. A + 0·1 cc. 50 % glycerol: the rest as before	Very slight

A similar result has been obtained in the case of the amylase from the American Cockroach, and would seem to indicate that the ability of the cockroach enzyme to function in a more acid medium is due in part at least to some associated substance which is not destroyed by heating at 100° for 5 minutes.

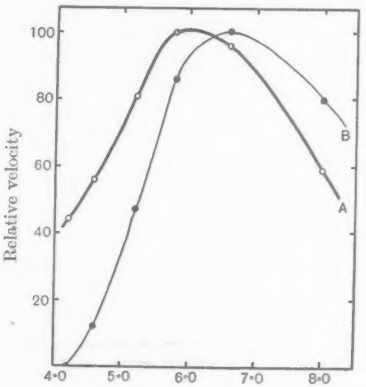

Fig. 4.

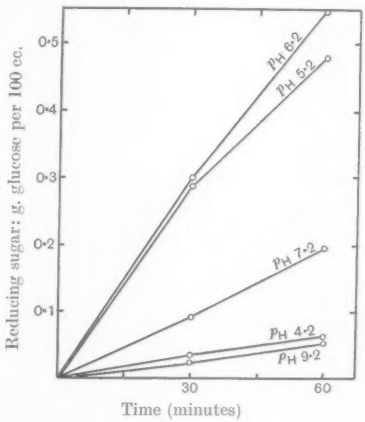

Fig. 5.

Fig. 4. p_H-activity curves (saccharogenic) for amylase. *A*, Salivary amylase of *Blatella germanica*. *B*, Human ptyalin.

Fig. 5. Hydrolysis of sucrose by cockroach invertase.

(iii) *Ratio of amyloclastic to saccharogenic activity.* Preparations of cockroach amylase and human ptyalin were adjusted to have about the same activity at p_H 6·6; and, preparing the mixtures in the usual manner, their amyloclastic and saccharogenic activities were compared at p_H 6·6 and 8·0. The ratio of amyloclastic to saccharogenic activity was arbitrarily expressed as $\frac{1}{t} : x$; where t is the time in minutes required to reach the arbitrary end-point of starch hydrolysis and x is the reducing sugar (expressed as g. "glucose" %) produced in 15 minutes. The results are shown in Table III.

Table III.

Enzyme	p_H	Ratio of amyloclastic to saccharogenic activity
Cockroach	6·6	1 : 2·5
,,	8·0	1 : 2·3
Human ptyalin	6·6	1 : 3·2
,,	8·0	1 : 3·3

Sherman and Schlesinger [1913] found that this ratio (expressed in other units) differed widely in various enzymes, ranging from 9 : 1 to 2 : 7. They found that whereas preparations of pancreatic amylase showed a more or less constant ratio, the ratio in the case of plant amylase preparations was extremely variable. Although a long series of experiments would be necessary to establish the point completely, the above results suggest that by this criterion human and cockroach amylases are similar in nature.

Invertase.

Of the enzymes which hydrolyse sucrose, that obtainable from yeast has been the most closely studied. This enzyme is reversibly inactivated by alkali, and the alkaline branch of the p_H-activity curve is regarded by Michaelis and Davidsohn [1911] as the dissociation-residue curve of a weak-acid enzyme which is active only in the form of undissociated molecules. The acid branch of the curve is similarly interpreted by Myrbäck [1926]; but in this region there is in addition the factor of irreversible destruction of the enzyme. The activity of yeast invertase is inhibited by heavy metals, by high concentrations of sodium chloride and by glycerol among other substances.

Euler and Svanberg [1921] studied the invertase of the human jejunum and found that it was active in a more alkaline medium than yeast invertase; and Euler and Myrbäck [1921] showed that this human invertase was more sensitive to heat.

Sarin [1923] observed that the invertase from the gut of the honey-bee was inhibited by glycerol. Nelson and Cohn [1924] made a careful comparison between the invertase of honey and of yeast. They found certain differences in the kinetics of the reaction in relation to substrate concentration, but the optimum concentration of sucrose for both enzymes was about 4 %. The optimal range of p_H was 3·5–5·0 in the case of yeast and 5·5–6·3 in the case of honey invertase, the same relative change in p_H having a greater effect upon the latter enzyme.

The distribution of invertase in the alimentary tract of the cockroach has already been described.

Methods.

The activity of invertase has been measured by the same method as the saccharogenic activity of amylase, 3 % sucrose being used as substrate. The concentration of enzyme has usually been adjusted to give rise to about 0·25 % reducing sugar (measured as "glucose") in 1 hour.

Under these conditions the hydrolysis of sucrose has been shown to be practically a linear function of time (Fig. 5), and the activity of the enzyme may be regarded as directly proportional to the amount of reducing sugar produced at the end of half an hour. This relation obtains around the optimal p_H of the enzyme and on the alkaline side of this; but on the acid side (see

p_H 4·2 and 5·2 in Fig. 5) the activity soon begins to diminish as the result of irreversible destruction of the enzyme and the relative activity will apparently fall off as the experiment proceeds. In other words, in short experiments with an active preparation the optimal zone will extend further in the acid direction. Hence the importance of so adjusting the concentration of the enzymes to be compared that they act at approximately the same rate.

Yeast invertase was prepared by grinding up fresh yeast with 50 % glycerol and a little fine sand. Human invertase was prepared by extracting for a week, with 50 % glycerol, the mucous membrane removed from the jejunum and then filtering.

The various preparations were incubated for 15 hours with a 0·5 % solution of glucose. There was no change in reducing power. None of the preparations contained glycolytic enzymes.

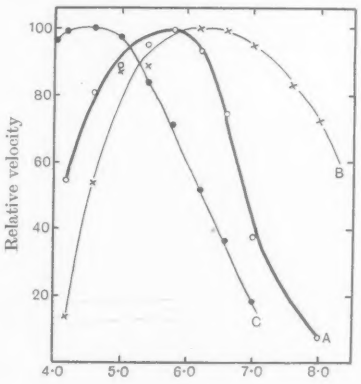

Fig. 6.

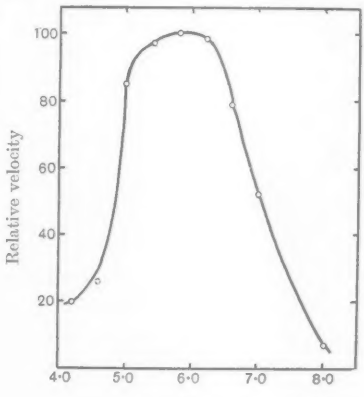

Fig. 7.

Fig. 6. p_H-activity curves for invertase. *A*, Mid-gut invertase from *Blatella germanica*. *B*, Invertase from human jejunum. *C*, Invertase from yeast.

Fig. 7. p_H-activity curve for invertase from the salivary gland of *Blatella germanica*.

(i) *The effect of salts, heavy metals and glycerol.* Low concentrations of sodium chloride have no effect upon the activity of cockroach invertase. A concentration of 1 % has an inhibitory action (Table IV).

Table IV.

Invertase from mid-gut of *B. germanica*.

NaCl concentration (g. %)	Relative activity
Nil	100
0·1	100
1·0	61

That glycerol when present in a concentration above 10 % has a strong inhibitory effect in spite of the increased quantity of enzyme present is shown in Table V.

Table V.

Glycerol extract of mid-gut of *P. americana*.

cc. of enzyme preparation	% glycerol	Relative activity
0·2	10	100
0·4	20	88

Cockroach invertase is extremely sensitive to silver nitrate. This is shown in Table VI.

Table VI.

Equal quantities of invertase (glycerol extract) from mid-gut of *B. germanica*.

Concentration of silver nitrate	Relative activity
Nil	100
0·0001 *N*	46·5
0·001 *N*	0

In all the above respects cockroach invertase behaves like yeast invertase.

(ii) *The effect of hydrogen ion concentration.* The methods employed were the same as those already described in connection with amylase. It was shown that at p_H 6·0 the activity was the same whether phosphate or phthalate buffer was used.

Fig. 6 shows p_H-activity curves for the invertase from yeast, from the cockroach and from the human jejunum. The curve for the cockroach enzyme is very similar to that obtained by Nelson and Cohn [1924] for honey invertase. The curves for yeast invertase and human invertase agree with those of Michaelis and Davidsohn [1911] and Euler and Svanberg [1921] respectively. It is clear that cockroach invertase is well adapted to work at the range of reaction (p_H 4·4–6·2) commonly found in the crop. As was noted by Nelson and Cohn in the case of honey invertase the zone of p_H over which it is active is more restricted than that of the other two enzymes.

At the end of this experiment the tubes were left in the water-bath for about an hour and then the p_H of the mixtures at 4·2 and 8·0 was adjusted to 5·8 by the addition of traces of 5 % caustic potash and of glacial acetic acid respectively (*vide infra* in connection with maltase). These mixtures were incubated for a further period of 30 minutes; and it was found that whereas the mixture previously at p_H 8·0 showed 100 % activity, that which had stood at p_H 4·2 was almost inactive, *i.e.* the enzyme is stable but inactive on the alkaline side of its optimal zone, while it is highly labile on the acid side—a behaviour identical with that of yeast invertase.

Fig. 7 shows the p_H-activity curve of invertase from the salivary gland of the German Cockroach. This curve falls off a little more rapidly in the acid direction but in other respects is the same as that described above.

Maltase.

Maltase has not been so extensively studied in the past as invertase. The maltase of yeast was shown by Michaelis and Rona [1913] to have a narrow p_H range of 6·1–6·8 and to be very sensitive to changes in reaction outside this zone, whether in the acid or the alkaline direction, and to be in general

a far more labile enzyme than invertase. It is readily adsorbed by kaolin, while invertase is not, and is readily extracted from this adsorbate with secondary alkali phosphate [Willstätter and Kuhn, 1921]. Its action is inhibited by glycerol and by sodium chloride above 0·2 *N* concentration. The properties of the maltase of the succus entericus do not seem to have been closely studied.

In the cockroach, maltase occurs only in the secretion of the mid-gut.

Methods.

Quantitative measurements of maltase activity have been made by the method of Hagedorn and Jensen (as described above), using 0·25 % maltose as substrate. By adding measured quantities of a standard solution of glucose to measured quantities of this substrate, a graph was prepared from which the reducing power of the mixtures in g. glucose %, as calculated from the table of Hagedorn and Jensen, could be converted into "percentage hydrolysis" of the maltose substrate. Serial experiments were usually continued until about 60 % hydrolysis had occurred in the fastest tube. In experiments on the p_H range of the enzymes, the p_H has been plotted against this "percentage hydrolysis." When such a large fraction of the substrate is hydrolysed, this percentage can give only an approximate indication of the "relative velocity" of action.

The first attempts to demonstrate maltase in the glycerol extracts of cockroach organs were unsuccessful. It seemed probable that the enzyme had been adsorbed by the ground sand used in the preparation. The extract was therefore poured off and the sediment shaken for about 2 hours at 30° with a few cc. of phosphate buffer p_H 6·8. This extract was very rich in maltase. In the experiment given in Fig. 8, for example, the enzyme equivalent to one-hundredth of a cockroach was present in each mixture and this effected 60 % hydrolysis of 1 cc. of 0·25 % maltose in an hour. Much of this activity was lost in the course of about a week at room temperature. The experiments recorded below were made upon such an extract or upon a similar extract made from the "dry preparations" of tissues.

For experiments on yeast maltase, an aqueous extract of dried yeast was employed. In the case of human maltase, the same preparation as for invertase was used, but this was suitably diluted to obviate the inhibitory action of the glycerol.

Table VII.

Test mixture	Percentage hydrolysis after 1 hour
No added salt or glycerol	37·0
0·1 % sodium chloride	37·5
1·0 % sodium chloride	8·0
10 % glycerol	0·0

(i) *The effect of sodium chloride and glycerol on cockroach maltase.* This effect is shown in Table VII. The action of maltase is partially inhibited by

1 % sodium chloride and completely inhibited by 10 % glycerol. It is more susceptible to glycerol than is invertase. In this it resembles yeast maltase; and this fact affords a further reason for the failure to demonstrate maltase in the glycerol extracts of cockroach tissues.

(ii) *Effect of hydrogen ion concentration on cockroach maltase.* Since the extracts containing maltase were made with phosphate buffer, special methods had to be adopted to obtain the required p_H. The experimental mixtures were made up as follows: 0·1 cc. of enzyme preparation, 0·2 cc. of buffer at the required p_H and 0·2 cc. of distilled water. A duplicate series of mixtures was made up in uniform tubes as follows: 0·2 cc. of the same series of buffers as before, and 0·3 cc. of distilled water. Suitable and identical amounts of appropriate indicators were added to the two series of mixtures; and the mixtures containing enzyme were adjusted to the same p_H as the standards without altering their volume appreciably in the following manner. The ends of two capillary tubes were drawn out into hair-like capillaries and filled with 0·5 *N* HCl and 0·5 *N* NaOH respectively, to such a level that when dipped in liquid the reagent would flow gently out. The test mixtures were stirred cautiously with one or other of these capillaries until the colour matched that of the standard. 0·5 cc. of 0·5 % maltose was then added and the experiment continued as previously described.

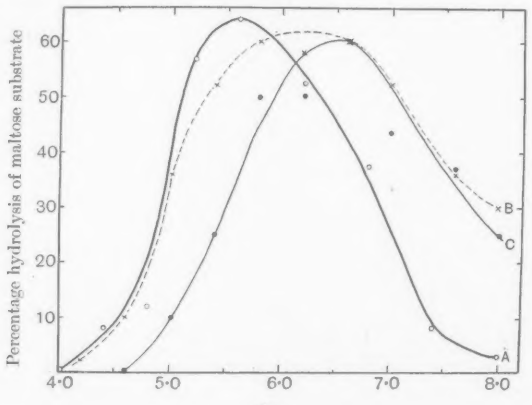

Fig. 8. p_H-activity curves for maltase. *A*, From mid-gut of *Periplaneta americana*. *B*, From human jejunum. *C*, From yeast.

Fig. 8 shows the "percentage hydrolysis" of maltose by the three enzymes plotted against p_H. These curves give only an approximate indication of the relative activity at the different reactions. The curve for cockroach maltase (with an optimal zone from 5·0 to 6·2) falls off more rapidly than that for invertase in the acid direction, but it is active over the greater part of the p_H range found normally in the crop of the insect. The curve for yeast agrees

well with that obtained by Michaelis and Rona [1913]. Human maltase is active in the acid direction as far as is the cockroach enzyme, but it is much more resistant than the latter on the alkaline side.

Lactase.

Relatively few comparative studies have been made upon lactase. The lactase of yeast is said to have its optimum p_H at 7·0.

The presence of lactase was demonstrated in extracts from the mid-gut of the cockroach, but not in those from salivary glands or crop. The formation of glucosazone and the increase in reducing power of a lactose solution, as measured by the method of Hagedorn and Jensen, were used as qualitative tests. The enzyme was obtained by extracting "dry preparations" of enzymes from the mid-gut with phosphate buffer at p_H 6·8. For the p_H-activity curve given in Fig. 9, the methods described under maltose were employed, both for the calculation of the percentage hydrolysis and for the adjustment of the p_H. The enzyme was far less active than the maltase in the same preparations. For example, in this experiment the enzyme equivalent of one-tenth of a cockroach was employed in each mixture and these were incubated for 15 hours. The range of p_H over which it is active is similar to that of the enzymes described above, the optimal zone being from 5·0 to 6·4.

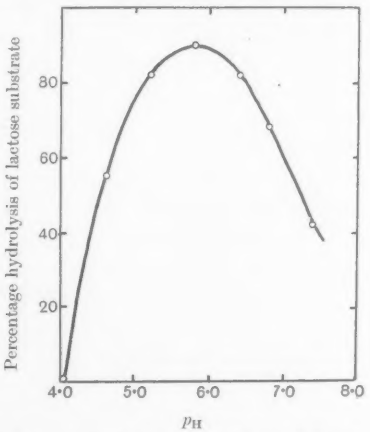

Fig. 9. p_H-activity curve for lactase from mid-gut of *Periplaneta americana*.

This lactase was shown to be completely inhibited by 10 % glycerol.

Neither the yeast preparations nor those from the human jejunum contained lactase.

Discussion.

Although, in the experiments here described, not all the known properties of the various enzymes have been compared, yet these experiments are sufficient to indicate the existence of a very close similarity between the

enzymes of the cockroach and those from yeast and from man. As was to be expected from the reaction of the crop contents of the cockroach, the enzymes of this insect are most active over an acid range from about p_H 5·0 to 6·5; and this range is similar for all the enzymes except amylase, in which it extends in the alkaline direction to p_H 7·0. It should be noted that the conditions under which this enzyme works are peculiar in that besides acting with the other enzymes in the crop, the saliva is expelled from the mouth of the insect and mixed with the food before it is ingested.

The results have been got from very small quantities of material; but the good agreement with the results of others, obtained when dealing on the same scale with the other enzymes, is evidence that they are reliable.

Summary.

1. The salivary glands of the cockroach secrete an amylase and in *Blatella germanica* (though not in *Periplaneta americana*) an invertase. Extracts of the crop, when thoroughly freed from food contents, do not contain enzymes acting on carbohydrates. The stomach and hepatic caeca secrete invertase, maltase and small quantities of lactase.

2. The amylase of the cockroach is inactivated by dialysis and is affected by salts in the same way as is human ptyalin. The ratio of amyloclastic to saccharogenic activity is similar to that of human ptyalin. The p_H-activity curves for these two enzymes have been compared. Under the same conditions cockroach amylase is more resistant to acid (optimum p_H 5·9). This is shown to be due in part at least to some associated factor which is not destroyed by heating at 100° for 5 minutes.

3. Cockroach invertase has been compared with yeast invertase and human jejunal invertase. It is partially inhibited by 1 % sodium chloride, 20 % glycerol and 0·0001 *N* silver nitrate; and completely inhibited by 0·001 *N* silver nitrate. The p_H-activity curve for cockroach invertase shows an optimal range from 5·0 to 6·2 which is intermediate between the ranges for yeast invertase and human jejunal invertase.

4. Cockroach maltase has been compared with the maltase of yeast and the human jejunum. It is partially inhibited by 1 % sodium chloride and completely inhibited by 10 % glycerol. It is adsorbed by powdered sand, but can be extracted from this with phosphate buffer p_H 6·8. The p_H-activity curve shows an optimal range from 5·0 to 6·2, which is more acid than the range in the maltase of either yeast or human jejunum.

5. Cockroach lactase is completely inhibited by 10 % glycerol and shows an optimal zone of p_H from 5·0 to 6·4.

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CVII. THE PRODUCTION OF LACTIC ACID IN FROG'S MUSCLE *IN VIVO*.

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(*Received June 29th, 1927.*)

Almost all the work which has been done upon the production of lactic acid in muscular activity (Fletcher and Hopkins, Meyerhof, etc.) has been carried out upon muscles removed from the animal body and consequently deprived of what must certainly be a potent factor in the recovery process *in vivo*, viz. the circulating blood. The experiments here described were carried out in order to see to what extent lactic acid produced by the activity of the living animal would accumulate in the muscles.

The work was begun in 1922 and as there is no prospect of our being able to complete the investigation we have considered it worth while to publish it in its present stage.

If a frog be placed in a large vessel of water at 35–40° it swims round with great violence and after a period of about 1 to 2 minutes it exhibits a jerky spasmodic movement of its legs as though the muscles were unable any longer to respond to the nervous stimuli being sent into them. Shortly after this stage all movement ceases and the frog becomes stiff and unconscious. But if, now, the animal be removed from the hot bath it will recover consciousness in a few minutes and gradually reacquire the power of movement in its limbs, though frequently the muscles do not appear to have recovered completely for at least half an hour. The various stages of this process were used for investigating the production and removal of lactic acid.

The following are typical observations made upon frogs under these conditions.

At 33°. Frog became weaker but could still swim about after being in the water up to 5 minutes.

At 35°. Frogs came to rest after 3 minutes and when taken out they seemed quite limp and toneless (cf. at 38°) until breathing and movements returned and then the tone seemed to return to the muscles all at once.

At 37°. Came to rest in 1 minute. Taken out at once. Eyes closed. Breathing started after 1 minute. Eyes slowly opened and breathing quickened. Legs not nearly so fixed as at 38° but still tend to return to a semi-extended position. Complete recovery after 2 minutes.

At 38°. (i) Frog swims about much more violently than at the lower temperatures tried. Stiff after 1 minute. Taken out at once. Legs stopped in a semi-extended position and returned to this point whether forcibly flexed or extended. Could first move legs very weakly after 2 minutes. Could first hop effectively after 4 minutes. Complete recovery in about 15 minutes.

(ii) Stiff after 1 minute. Left in bath 30 seconds longer. Breathing recommenced after about $1\frac{1}{2}$ minutes. Reflexes in hind legs began to appear after $3\frac{1}{2}$ minutes but could not be repeated at short intervals. Front legs remained stiff. Remained very sluggish for upward of 20 minutes. Breathing as in the normal frog during this time. Could not jump properly. Could not execute many movements properly. At end of 2 hours recovery in all limbs was complete and frog appeared normal in every way.

The observations on the lactic acid content of the muscles have been made only at 38°, the frog being left in the bath in each case until stiff, *i.e.* about 1 minute. The lactic acid was estimated by the micro-method of Meyerhof [1920], the muscle being removed as rapidly as possible from the hind legs of the frog used and one estimation made on each leg.

Procedure	g. lactic acid per 100 g. muscle	
"Resting minimum" of normal frog...	0·031	0·031
Muscle removed from frog and kept at 40° for 1 hour. ("Lactic acid maximum")	0·406	—
Muscle removed from frog and kept at 40° for 1 hour. ("Lactic acid maximum")	0·443	—
Pithed frog in bath at 38° for 1 min. 30 secs.	0·042	0·044
Active frog left in bath at 38° until stiff	0·112	0·125
" " "	0·125	—
" " "	0·112	0·113
" " "	—	0·176
" " "	—	0·125
Frog in bath as above, after recovery at room temperature for 18 minutes	0·107	0·111
" " " " 20 "	0·091	0·096
" " " " 32 "	—	0·070
" " " " 30 "	0·039	0·045
" " " " 60 "	0·037	0·040

It will be seen that at the time when the animal is unable any longer to bring about the contraction of its muscles, the latter contain a high percentage (average 0·125 %) of lactic acid. Meyerhof [1920] showed that the gastrocnemius of the frog, removed from the body and stimulated indirectly by means of a tetanising current until it could no longer respond, attained a concentration of lactic acid of about 0·19 %. Hence it is not improbable that the movements of the frog are arrested by the lactic acid accumulating under the conditions of our experiments more rapidly than it can be removed.

If the frog is pithed and left in the hot bath for a corresponding period, there is very little more lactic acid present than the normal resting minimum (0·043 % as against 0·031 %). The lactic acid is, clearly, the result of muscular activity.

The figures also show that the removal of this accumulated lactic acid is very slow. It is often not completely removed in half an hour, though after the lapse of an hour the resting minimum value has been practically regained.

The large lactic acid production and the slowness of the recovery process under these conditions should make this material very suitable for studies on lactic acid in the living organism.

SUMMARY.

1. When placed in a bath at 38° the frog is at first violently active, but becomes stiff and motionless in about 1 minute.

2. In this state the muscles of the hind legs show an average content of lactic acid of 0·125 %.

3. The muscles of a pithed frog under similar conditions contain little more lactic acid (0·043 %) than the normal resting minimum (0·03 %).

4. On removal to room temperature the frog recovers in a few minutes, but the lactic acid content of the muscles does not regain the normal level until half to one hour later.

We wish to express our thanks to Sir F. G. Hopkins for suggesting this problem to us and for his helpful interest in the work. The research was carried out during the tenure by each of us of a Frank Smart Studentship of Gonville and Caius College and of a grant from the Board of Scientific and Industrial Research.

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CVIII. THE TITRATION OF PROTEIN HYDROLYSATES.

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Foreman [1920] and Harris [1924] have shown that the acid radicles of amino-acids can be titrated quantitatively if the K_a of such amino-acids in solution were increased sufficiently to afford a sharp reaction with the indicators used. This was effected by titrating in strong alcoholic solution (above 87 % alcohol) to phenolphthalein in Foreman's method and to thymolphthalein in Harris's method. Better results were obtained by the addition of formaldehyde to the alcoholic solution according to Sørensen's method [1907]. Also, by the inclusion of formaldehyde in the solutions, difficulties in obtaining accurate results with dicarboxylic acids and diamino-acids were overcome.

The procedure was to neutralise the *aqueous* solutions to the respective indicators and, after adding the requisite volume of neutralised solvent to the solution, again to neutralise to the indicator used. This increase in acidity was the basis for calculating the acid radicle present in the solutions. Work was done on most of the common amino-acids individually so that data of the behaviour of all the different types of amino-acids under such conditions have been collected.

Attention has been paid in the present work to the actual distribution of nitrogen in solutions containing mixtures of amino-acids and not to the total acid radicle present. The principle of weight of nitrogen equivalent to titratable acid radicle has, therefore, been considered. With a solution of glycine hydrochloride, the hydrochloride portion would be neutralised completely in aqueous solution with alkali to phenolphthalein, whereas in the subsequent alcoholic titration, the carboxyl group would be quantitatively neutralised. The titre of the carboxyl group would be exactly equivalent to the weight of nitrogen present in the glycine solution, *i.e.* every 1 cc. of normal alkali used in the alcoholic titration would be equivalent to 0·014 g. of nitrogen.

From Foreman's paper [1920] the following deductions have been drawn concerning the nitrogen of the various amino-acids equivalent to the titratable value of such acids in alcohol-formalin solution after previous neutralisation in aqueous solution:

Monoamino-acids.	All the nitrogen.
Dibasic acids.	All the nitrogen (one carboxyl group is neutralised in aqueous solution).
Cystine and lysine.	All the nitrogen.
Arginine.	One-quarter of the nitrogen.
Histidine.	One-third of the nitrogen.

Ammonium salts enter into the same class as monoamino-acids.

With the above data, the alcohol titration value of a mixture of amino-acids in solution could be calculated, even if the nitrogen distribution (Van Slyke) only were known (provided, of course, that the various amino-acids did not react together in alcohol-formalin solution, thus upsetting the actual titration values of the amino-acids collectively); *e.g.* if the distribution of nitrogen in the diamino-acids plus cystine fraction in the Van Slyke method were correct for each amino-acid, the titration value of that solution could be calculated.

Work on protein hydrolysates.

A. *Titration values of complete hydrolysates compared with the sum of the titration values of the fractions separated by the Van Slyke method.*

Gelatin. 18 g. of pure gelatin were hydrolysed with 20 % hydrochloric acid for 36 hours and the hydrolysate evaporated twice to dryness *in vacuo*. The gummy residue was dissolved in water and decolorised with a small amount of Merck's medicinal charcoal and made up to a litre. 150 cc. were proceeded with in the ordinary Van Slyke method of separating into groups. The phosphotungstates of the diamino-acids plus cystine were decomposed by the ether-amyl alcohol method, the solution of amino-acids being freed as much as possible from hydrochloric acid and made up to 100 cc. Excess of phosphotungstic acid was removed from the filtrate by ether-amyl alcohol and the solution freed from as much hydrochloric acid as possible and made up to 250 cc. Alcohol-titration values of the original solution, filtrate, diamino-acid plus cystine fraction, and the equivalent of the amide nitrogen were determined.

Caseinogen (commercial sample 1). The same procedure as for gelatin was carried out for 18 g. of a sample of commercial caseinogen. The alcohol titration values of the original solution, the filtrate, the diamino-acid plus cystine fraction, and the equivalent of the amide nitrogen were determined.

Table I. *Gelatin and caseinogen hydrolysates. Comparison of the alcohol-titration values of the complete hydrolysates against those of the fractions separated by the Van Slyke method.*

	Gelatin	Caseinogen
Total nitrogen (g.)...	0·4644	0·4245
Titration value (cc. 0·1 *N* alkali) of complete hydrolysate	271·8	253·9
Filtrate	218·8	172·0
Diamino-acids plus cystine ...	44·7	52·3
Equivalent of amide-N	7·5	30·4
Total of fractions	271·0	254·7
Titration equivalent as g. nitrogen	0·3794	0·3566
Nitrogen not titrated (g.)... ...	0·0850	0·0679
„ „ (% total nitrogen)	18·30	16·00

From Table I it can be seen that the sums of the titration values of the fractions were almost identical with the titration values of the original solutions. Hence, there has been no change in the titration values of the hydrolysates effected by the process of separating the fractions by the Van Slyke method.

B. *The titration values of the diamino-acid plus cystine fractions of various proteins.*

Three separate samples of caseinogen, I and II being commercial samples and III a pure sample, were investigated, each in duplicate, for distribution of nitrogen according to the Van Slyke method. The gelatin cited in A above was also studied in the same direction in duplicate (150 cc. per determination). During the course of the author's work [Davies, 1927] on the proteins of green forage plants, eight samples of proteins were prepared from green brassicaceous plants and two from umbelliferous plants and analysed by the Van Slyke method in duplicate. For each of the above proteins the different amounts of nitrogen allotted to arginine, histidine, lysine and cystine were worked out in the usual way. Part of the diamino-acid solution in each determination was used to determine the titration value in alcohol-formalin solution (after previous neutralisation of the aqueous solution to phenolphthalein).

From the amounts of nitrogen allotted in each case to the different amino-acids (not corrected for the solubilities of the bases) the theoretical titration value was calculated as cc. 0·1 *N* alkali (*i.e.* one-fourth arginine-N, one-third histidine-N, total lysine- and cystine-N ÷ ·0014). The results obtained are given in Table II.

Table II. *Calculated and actual alcohol-titration values of the diamino-acids (Van Slyke method) of various proteins.*

Source of protein	N per determination (g.)	Alcohol-titration values of diamino-acid fraction as cc. 0·1 *N* alkali		Calculated: found
		Calculated	Found	
Caseinogen I	0·4245	41·36	52·30	1·27
Caseinogen II	0·4511	35·79	44·10	1·23
Caseinogen III	0·4674	47·64	57·90	1·22
Gelatin	0·4644	36·36	44·74	1·23
Cabbage I	0·4372	42·00	51·35	1·25
Kohlrabi	0·4263	41·65	49·78	1·20
Marrow stem kale leaves	0·4089	41·71	50·34	1·21
Ditto. Stems	0·4576	42·71	54·24	1·27
Turnip leaves	0·5355	53·10	63·08	1·19
Turnip root	0·4750	51·07	60·52	1·19
Cabbage II	0·4167	35·57	43·63	1·23
Turnip leaves II ...	0·3906	31·79	36·84	1·16
Carrot	0·3814	30·93	39·30	1·23
Parsnip	0·4286	42·15	50·00	1·19
				Average = 1·22

(The Van Slyke distributions of nitrogen were determined in duplicate for each of the above proteins, the average values only being used in compiling Table II.)

Experimental Note. *N*/10 aqueous alkali was used for the aqueous neutralisation and *N*/25 alcoholic potash for the alcoholic titration. 10 cc. (*ex* 100 cc. of diamino-acid solution) required from 9 to 12 cc. alcoholic potash for the titration.

C. *Titration of the monoamino-acid fraction (the filtrate of the Van Slyke method).*

The monoamino-acid fractions for most of the proteins of Table II had been treated with caustic soda and hydrochloric acid and were, therefore, unsuitable for amino-acid titration owing to separation of salts from alcoholic solution. Samples of filtrate from gelatin and caseinogen I were treated with ether-amyl alcohol to get rid of the excess phosphotungstic acid used to precipitate the bases. The solutions were evaporated to dryness twice and made up to a known volume, the titration values of aliquot portions of these being then determined (*v.* Table III).

Table III. *Titration of the specially treated monoamino-acid fractions of protein hydrolysates (obtained by the Van Slyke method).*

Protein	Total nitrogen in hydrolysate (g.)	Nitrogen in filtrate (g.)	Titration value cc. $N/10$ alkali: Calculated	Found	Calculated: found
Gelatin	0·4245	0·3328	237·7	226·3	0·95
Caseinogen I	0·4644	0·2608	196·3	172·0	0·92

EXPERIMENTAL NOTE. $N/10$ alcoholic potash was the strength of alkali best suited for the titration. 10 cc. (*ex* 250 cc. total solution of monamino-acids) requiring 6 to 7 cc. $N/10$ alkali for the alcoholic titration.

SIGNIFICANCE OF THE VALUES DETERMINED BY ALCOHOLIC TITRATION.

From Table I it is apparent that there has been no change in the titration values of the hydrolysates due to the processes of separating the fractions, and that losses during the operations were negligible. From Table II the titration values found for the diamino-acid solutions were uniformly greater than the expected values as calculated from the figures obtained by the Van Slyke method by 22 % of the latter.

Table III shows that the increase in titration apparent in Table II is balanced by the filtrate showing a lower titration value than that calculable for a monoamino-acid solution (a decrease of 8 % on the average). Theoretically, this solution should contain no nitrogen not accounted for by titration. As it is, the gelatin filtrate contained 0·016 g. and the caseinogen 0·020 g. not accounted for in the titration. These nitrogens, undoubtedly, belong to the bases, arginine and histidine, being the amounts not precipitated by phosphotungstic acid. Plimmer and Rosedale [1925] have advised the determination of arginine in the original hydrolysate, since its determination in the phosphotungstic precipitate gives low values. This fact is supported in this work. Van Slyke [1915] corrects for the solubility of the bases by adding 0·0032 g. nitrogen to the arginine nitrogen and 0·0038 g. to the histidine nitrogen, the total corresponding to 0·0050 g. of *untitratable* nitrogen in the filtrate. From the present work it is obvious that these corrections are too low. Plimmer and Rosedale have made similar deductions.

The relation of the titration values to the amino-nitrogen.

For the diamino-acids the titration values should be equivalent to the amino-nitrogen. That is, the equivalent of the sum of the lysine and cystine nitrogen, a third of the histidine nitrogen and a quarter of the arginine nitrogen should equal the titration value of the bases in alcoholic solution. Table II, columns 2 and 3, shows the calculated and observed values for the series of proteins. The equivalent found by titration is uniformly 22 % higher than the calculated value. Plimmer [1924] has observed the unsatisfactory results obtained by the method prescribed by Van Slyke [1915] for the determination of the amino-nitrogen of the bases. The specified time for reaction with nitrous acid is not sufficient for all the amino-nitrogen, especially that of lysine, to react and Plimmer has introduced longer periods than 5 minutes' shaking so as to get more accurate results. The results for the series of proteins (Table II) were obtained by the original Van Slyke method, and this discrepancy between calculated and observed values bears out Plimmer's observations. The uniformity of the discrepancy also seems to point out that, irrespective of the composition of the phosphotungstic precipitate, only about 82 % of the amino-nitrogen reacts during the 5 minutes' shaking. (The actual discrepancy cannot, of course, be calculated since the only basis of calculation is afforded by incorrect results.)

It is tentatively suggested, therefore, that alcohol-titration values of the phosphotungstic fraction converted into g. nitrogen give a more accurate value for the amino-nitrogen of that fraction.

In the filtrate fraction the titration value conveys nothing as to the exact state of the nitrogen in the monoamino-acids, since the nitrogen of a monoamino-acid titrates quantitatively whether it is amino- or imino-nitrogen.

Other sources of error in the Van Slyke method.

It is well known that during the Kossel and Kutscher method of determining the diamino-acids when lysine is precipitated from the arginine- and histidine-free solution, there is more nitrogen present in the phosphotungstic precipitate than can be precipitated as lysine picrate even when the picrate is crystallised out in three or four successive isolations. There is no doubt that phosphotungstic acid precipitates small but appreciable amounts of monoamino-acids under such conditions. There is the same possibility in the Van Slyke method that some of the nitrogen precipitated is monoamino-acid nitrogen. Such nitrogen titrates quantitatively in alcoholic solution and is accounted for under lysine nitrogen in the distribution table. The amount of imino-monoamino-nitrogen is comparatively small in most proteins, and its possible precipitation would account for only a small fraction of the discrepancy between the results for observed amino-nitrogen and the titration equivalent of the amino-nitrogen of the bases mentioned above. Titration values throw no light in this direction.

Suggested modifications of the Van Slyke method for determining the distribution of nitrogen in seven groups.

In view of the above work the following modifications of the Van Slyke method are tentatively suggested.

The complete hydrolysate. The arginine nitrogen is determined by the Plimmer method [1916] in an aliquot portion of the *original* hydrolysate, allowing for amide nitrogen driven off in the same process. The difference between the total nitrogen of the complete hydrolysate and the nitrogen equivalent to the alcohol-titration value of the same liquid is the non-titratable nitrogen and is equal to three-fourths of the arginine nitrogen plus two-thirds of the histidine nitrogen. From these two values the true arginine and histidine nitrogen values are obtained.

The phosphotungstic precipitate. The separation of the bases is carried out in the usual manner, the precipitate decomposed by the ether-amyl alcohol method and the alcohol titration value determined. The precipitated arginine and cystine are determined in the usual way, as well as the total diamino-nitrogen, so as to calculate the lysine nitrogen. The titration value converted to its equivalent weight of nitrogen is taken as the amino-nitrogen of the phosphotungstic precipitate. The precipitated histidine is calculated from the above data and the lysine determined by difference. The amounts of the precipitated arginine and histidine nitrogens are subtracted from the true values obtained from the complete hydrolysate and the differences, properly distributed into amino- and non-amino-nitrogen, allowed for in the two classes of nitrogen in the filtrate. The true arginine and histidine values are, of course, not corrected for solubility, and neither is the lysine nitrogen. Cystine nitrogen should be corrected for solubility according to the amounts laid down by Van Slyke [1912], but the amount of cystine present in ordinary proteins is too small for accurate determination by the distribution method, and no great stress can ever be put on the accuracy of this group.

The filtrate. This is best prepared for amino-nitrogen determination in the same way as the phosphotungstic precipitate, that is, the filtrate and washings from the precipitate are shaken up three times with ether-amyl alcohol mixture, the mixture being finally washed twice with dilute acid. The extracted solution is then evaporated to dryness *in vacuo* at 40°, dissolved in water and made up to 250 cc.

(Owing to nitrogenous impurities in some samples of amyl alcohol it will be found convenient to wash the half-and-half mixture of ether and amyl alcohol twice in a separating funnel with 5 % hydrochloric acid, or the mixture can be stored over 5 % acid and siphoned off as required. The nitrogenous bases, even in commercial amyl alcohol, can be completely removed by this method.)

The filtrate by this treatment can be titrated so as to check the titration value of the amino-acids precipitated by phosphotungstic acid. The quota of untitratable nitrogen in the filtrate can also be calculated and allowed for.

Practical results from the method.

Table IV sets out the results for gelatin as determined by the titration method compared with those obtained by the original Van Slyke method.

Table IV. *Results for gelatin by the titration method compared with those by the original Van Slyke method.*

	Percentage of total		
Nitrogen	Arginine	Histidine	Lysine
Titration method	17·23	2·58	5·71
Van Slyke method	14·70	4·48	6·32
	Percentage of protein		
Amino-acid	Arginine	Histidine	Lysine
Titration method	9·63	1·72	2·61
Van Slyke method	8·22	2·99	2·89
Kossel and Kutscher	9·3	0·4	2·8

For gelatin, closer values to the Kossel and Kutscher results are obtained by the modified method than by the original Van Slyke method.

SUMMARY.

Protein hydrolysates can be titrated to phenolphthalein in neutralised formalin-alcohol solution. The sum of the titration values for the amino-acid fractions separated by the Van Slyke method is equal to the titration value of the original hydrolysate.

By working in terms of nitrogen equivalent to the titratable value of solutions of amino-acid mixtures, much information can be obtained concerning the nitrogen distribution in such solutions.

The equivalent of the nitrogen of the monoamino-acid fraction should be quantitatively the same as the alcohol-titration value, but it has been found not to be completely represented by the titration value, this untitratable nitrogen representing three-quarters of the arginine and two-thirds of the histidine nitrogen not precipitated with phosphotungstic acid during the separation.

The amino-nitrogen of the diamino-acid fraction should be the equivalent of the titration value, but the titration equivalent has been found to be uniformly 22 % higher than the amino-nitrogen values determined by the nitrous acid method.

A modification of the Van Slyke method for determining the distribution of nitrogen in seven groups is suggested. This entails calculating the true values of the arginine and histidine nitrogens in the original hydrolysate, and the use of the titration value of the phosphotungstic fraction for calculating the amino-nitrogen content of that fraction.

In the practical application of the method, results more closely agreeing with the values found by the Kossel and Kutscher method are given for the diamino-acids.

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CIX. VARIATIONS IN THE p_H AND BICARBONATE OF THE PLASMA AND OF THE ALVEOLAR CO_2 DURING FORCED BREATHING.

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Introduction.

The production of a rise in the p_H of the blood in voluntary hyperpnoea has been inferred by Davies, Haldane and Kennaway [1920] and by Collip and Backus [1920] as the result of observations made on the blood and urine during forced breathing. These observers found that the titratable acidity of the urine was decreased, and thus confirmed the experimental work of Leathes [1919].

Collip and Backus also estimated the alveolar CO_2 and the bicarbonate of the plasma, which they found were always reduced, and calculated the p_H of the blood from Hasselbalch's formula. They found that the hydrogen ion concentration of the blood was lowered.

Haldane investigated the CO_2-absorption curve of blood drawn at the end of an hour's forced breathing, and found that it was unaltered. The changes produced in the partition of the available base between the plasma and corpuscles by a lowered tension of CO_2 must therefore be transient and depend only on the alveolar CO_2 tension. He calculated that the p_H of his blood had been raised 0·38 in the course of the experiment.

Grant and Goldman [1920] using the dialysis method of Levy and Rowntree, and Peters and his colleagues [1926] using Cullen's [1922] colorimetric method, made direct observations on the p_H of the blood during hyperpnoea, and found an increase of 0·1 to 0·2 p_H above the normal.

Observations at frequent intervals have not however been carried out. The micro-method of Martin and Lepper [1926, 1] furnished us with a technique whereby we could take successive readings at intervals of a few minutes, and so study in detail the changes which take place as a result of reducing the tension of CO_2 in the alveolar air.

In some preliminary experiments on forced breathing, observations were confined to the rate of rise and the return to normal of the p_H of the blood, samples being taken at intervals of 3 minutes. Estimation of the percentage of CO_2 in the alveolar air at different rates of respiration, combined with observations on the changes which occurred in the p_H of the blood, were then

made. These experiments showed us the most effective rate of respiration for the subject concerned. At the same time we found out certain precautions that had to be taken in the collection of approximately simultaneous samples of blood and of alveolar air respectively. The limits of endurance of the experimental subject (E. H. L.), who had to be responsible for the collection of blood, was also ascertained. The symptoms experienced were a feeling of chilliness and slight mental confusion, which came on within a few minutes. Increased salivation was accompanied by a troublesome cough towards the end of the experiments. Tetanic spasms were never experienced, slight rhombergism was nearly always present, and in long experiments, twitching of the muscles of the face accompanied by shakiness of the hands. During recovery the longest period of acapnoea noted was $1\frac{1}{2}$ minutes. The rapid rate at which the mental symptoms came on was a constant surprise. By the end of 2 minutes it was difficult to concentrate sufficiently to count the respirations with a stop-watch. To the onlooker the effect was comparable with the early stages of alcoholic intoxication. Irritability, unreasonableness with a tendency to argue, and a lowering of the critical faculty were marked. Nevertheless, no difficulty in collecting the samples of blood was experienced so long as the routine was not departed from. The rate of respiration, once established, became almost automatic. We therefore arranged all the details of an experiment beforehand, and the subject took samples of blood only when ordered to do so and was not responsible for timekeeping. We found that we could be certain of carrying on an experiment for 25 to 30 minutes, but that after that time symptoms such as cough or shakiness of the hands might interfere with the collection of samples.

Methods employed.

The p_H *of the blood* was estimated by the Martin-Lepper [1926, 1] method. Capillary blood was collected from the finger. The saline with which the blood was diluted was adjusted to p_H 7·60 for the normal observations and to 7·75 for the forced breathing experiments.

The alveolar CO_2 was estimated with a small Haldane-Priestley apparatus. Owing to the limited time at our disposal we could only make single observations. The sample of alveolar air was always taken at the end of a forced expiration.

The bicarbonate of the plasma was estimated by the method of Van Slyke [Van Slyke, Stillman and Cullen, 1919; Van Slyke, 1922] in which excess of acid is added to a measured amount of plasma and the mixture titrated back to the discovered p_H of the blood as drawn.

The plasma was collected by pricking the ear and running the blood down a capillary tube into a glass capsule containing liquid paraffin with a small amount of solid potassium oxalate to prevent clotting. The corpuscles were spun down as soon as possible and the plasma pipetted off into a tube coated with solid paraffin. Some preliminary experiments with rabbits' blood showed

us that the titration value of the bicarbonate was the same whether the cells were separated at 38° in the hot room or the blood was allowed to cool for a few minutes and then centrifuged at room temperature. We had to rely on the presence of oxalate to prevent glycolysis between the withdrawal of the blood and the separation of the cells from the plasma, it being impossible to use unneutralised solid sodium fluoride without lowering the alkali content of the blood. In view of the rapid glycolysis of blood in which the CO_2 tension has been reduced [Evans, 1922] we were doubtful as to whether the antiglycolytic action of oxalate were sufficient for our purpose, but a control experiment failed to detect any error from this cause over a period such as was used in the experiments.

The bicarbonate of the urine was estimated by Haldane's method [Davies, Haldane and Kennaway, 1920] in which excess of acid is added to 10 cc. of urine, the CO_2 shaken out and the mixture titrated to p_H 7·60.

The conditions of the experiment.

The forced breathing was carried out from $\frac{1}{2}$ to $2\frac{1}{2}$ hours after meals. The majority of the experiments took place about an hour after lunch. Half an hour after that meal the subject took two samples of blood for the p_H estimations and then gave a sample of alveolar air into the Haldane pipette. It is important to observe this sequence, as it is difficult when apnoeic to avoid cessation of respiration whilst blocking the mouthpiece of the sampling tube with the tongue. Blood was then collected for the bicarbonate estimation. The results of these observations gave the values for the subject when breathing naturally.

The experiment was then started. The respirations were maintained at 40 to 44 per minute, forced inspiration and expiration being carried out. In those experiments in which the p_H and bicarbonate of the plasma and the CO_2 of the alveolar air were all estimated, the following order was observed. After a period of 5 minutes' forced breathing, blood was taken for the p_H estimations, then a sample of alveolar air, then blood for bicarbonate, and further samples in the same order as soon as the gas analysis had been completed.

Results.

Normal conditions.

The values obtained for p_H, alveolar CO_2 and plasma bicarbonate under normal conditions are given in Table I. p_{K_1} has been calculated from Hasselbalch's formula, after subtracting 0·22 from the p_H observed at 18° to obtain the value for the p_H of plasma at 38° [Martin and Lepper, 1926, 2]:

$$p_{K_1} = p_H^{38°}\ 38° \log \frac{\text{plasma bicarbonate}}{\text{CO}_2 \text{ dissolved in plasma at } 38°}.$$

Table I.

Date (1927)	p_H (mean)	Alveolar CO_2 %	CO_2 dissolved in plasma, 38° vols. %	Plasma bicarbonate vols. % CO_2	p_{K_1}
21. iii.	7·61	6·00	3·25	51	6·195
21. iii.	7·605	6·15	3·35	48	6·229
24. iii.	7·66	5·97	3·25	54·4	6·217
24. iii.	7·68	6·03	3·3	56	6·231
24. iii.	7·678	6·03	3·3	52	6·260
26. iii.	7·667	5·90	3·2	51	6·245
26. iii.	7·678	5·85	3·17	56	6·211
7. iv.	7·65	5·95	3·23	48	6·258
10. iv.	7·607	6·05	3·3	49·75	6·209
15. iv.	7·635	5·87	3·2	45·5	6·263
24. iv.	7·624	6·11	3·35	46	6·264
25. iv.	7·629	6·04	3·3	41	6·315
					Mean = 6·241

Observations during forced breathing.

1. *The* p_H *of the blood* showed a rapid rise reaching in 10 to 15 minutes a maximum of 0·2 to 0·24 above the initial value, at which level it was maintained as long as observations could be carried out. As soon as the forced breathing was stopped the p_H fell rapidly.

2. *The percentage of alveolar* CO_2 was halved at the end of 5 minutes, this rapid fall being followed by a further gradual decrease. The return to normal is shown in Figs. 2 and 3.

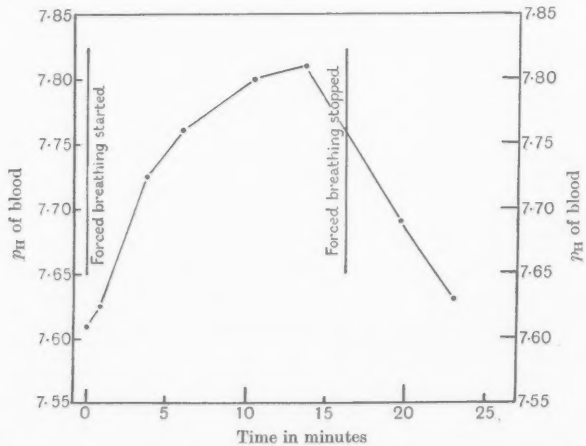

Fig. 1. Showing the rise in the p_H of plasma during forced breathing and the return to normal.

• — • — • p_H of blood.

3. *The bicarbonate of the plasma* was reduced. The titration of the plasma after forced breathing afforded results corresponding to 10 to 12 volumes % less total CO_2 than before the experiment was started. This drop was caused in two ways. The diminished CO_2 tension in the alveoli of the lungs accounts in

different experiments for 5 to 8 vols. %. The balance, 4 to 5 vols. %, is due to the higher p_H to which the plasma had to be titrated, sometimes as high as 7·85. More than the normal amount of sodium was in combination with the blood proteins, for it was found that, for the blood used, the titration value

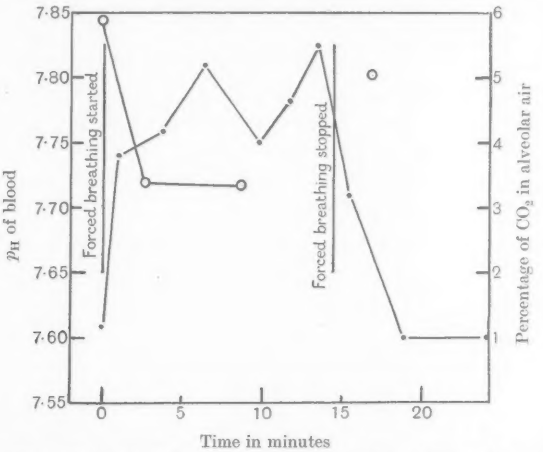

Fig. 2. Showing the rise in the p_H of the plasma and the fall in alveolar CO_2 during forced breathing The somewhat low values for p_H at 4 and 10 minutes are due to the sample of alveolar air having been taken immediately before the p_H estimation.

• —— • —— • p_H of blood.
○ —— ○ —— ○ Percentage CO_2 in alveolar air.

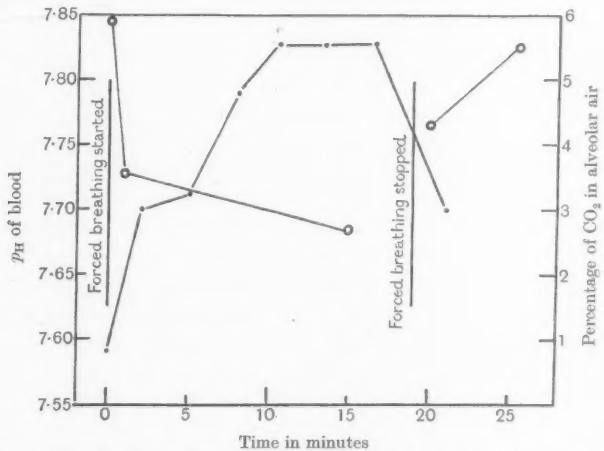

Fig. 3. Showing the rapid return to normal of the alveolar CO_2 when forced breathing is discontinued.

• —— • —— • p_H of blood.
○ —— ○ —— ○ Percentage of CO_2 in alveolar air.

of the plasma bicarbonate corresponded to 4 vols. % less CO_2 at p_H 7·80 than at p_H 7·60.

4. *Changes in the urine* were studied in two experiments. In both cases the urine became alkaline, containing 0·019 *M* and 0·036 *M* bicarbonate respectively. In the course of an experiment which lasted for 1 hour 0·357 g. of bicarbonate was excreted.

Fig. 4 gives a résumé of a typical experiment. It will be seen from Fig. 4 that the p_H of the blood and the alveolar CO_2 are changing continuously throughout the period of the observations. The values of p_{K_1} have therefore been calculated by interpolation from charts of the experiments, as it was impossible to estimate all three factors simultaneously. The results of a number of experiments are shown in Table II.

Table II.

Date (1927)	Duration of experiment (mins.)	p_H (mean)	Alveolar CO_2 %	CO_2, vols. % dissolved	Plasma bicarbonate vols. % CO_2	p_{K_1}
7. iv.	14	7·845	2·65	1·45	36	6·231
10. iv.	20	7·830	2·48	1·37	40	6·150
15. iv.	10	7·771	2·80	1·55	35	6·200
15. iv.	15	7·815	2·60	1·42	34·5	6·210
15. iv.	20	7·860	2·40	1·30	34	6·223
15. iv.	23	7·862	2·24	1·23	34	6·200
15. iv.	10	7·775	3·00	1·65	35	6·229
15. iv.	15	7·790	2·75	1·50	35	6·202
						Mean = 6·213

Discussion of results.

The experimental error with micro-methods is necessarily high, and when a constant depends on the value of three different micro-estimations, the errors of which may be cumulative, the results cannot agree very closely. The experimental error on the p_H observations was probably not more than $\pm$ 0·02, on the alveolar CO_2 $\pm$ 2·5 %, on the bicarbonate titration $\pm$ 1·5 %. If in any experiment a summation of errors occurred, the variation of the constant might be $\pm$ 0·08 on p_{K_1}. The mean of a number of experiments should, however, give a fair approximation to that constant. The value 6·24 obtained for p_{K_1} at 38° under normal conditions is higher than the values obtained for human blood by Warburg, 6·18, or by Parsons, 6·19 [Warburg, 1922]. These observers did not, however, add any fluoride to prevent glycolysis, and their experiments frequently lasted for 24 hours or more.

On the other hand, our bicarbonate estimations are probably slightly too low, as the solid potassium oxalate used raised the tonicity of the plasma and abstracted water from the corpuscles. The recent experiments of Eisenman [1927] would suggest that the error from this cause was not more than 1 %. Further, owing to the conditions of our experiments we could take only one sample of alveolar air, and we chose the expiratory sample.

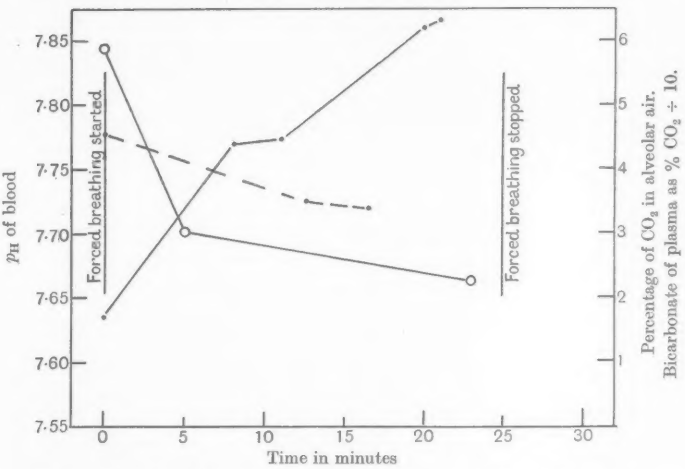

Fig. 4. The p_H and bicarbonate of the plasma and the alveolar CO_2 during forced breathing.

• —— • —— • p_H of blood. ○ ——— ○ ——— ○ Percentage of CO_2 in alveolar air.
— — — Bicarbonate of plasma.

Moreover, the correction factor of 0·22, which we have used for calculating the p_H at 38° from the observed value at 18° may be too low. Marrack and Thacker [1926], with a number of different bloods at the two temperatures, found a mean value of 0·247 for this correction factor.

These four considerations would all have the effect of making our value for p_{K_1} higher than that of previous observers.

The constants we obtained under normal conditions and during forced breathing are, however, comparable, and do not differ more than can be accounted for by the experimental error. We conclude therefore that in hyperpnoea carried out within 2 hours after a meal the rise in the p_H of the blood corresponds to the alteration found in the concentration of bicarbonate in the plasma and in the tension of CO_2 in the alveoli respectively.

The true nature of the "alkalosis" produced in forced breathing has been pointed out by Grant and Goldman [1920] who drew attention to the fact that, although the p_H of the blood was raised, the bicarbonate of the plasma was considerably reduced, their experiments showing an average reduction of 14 % less total CO_2. It would appear that the rise in p_H protects the body from loss of bicarbonate.

A slight definite diminution in the amount of base in the body does, however, occur during forced breathing, as evidenced by the amount of bicarbonate excreted in the urine. In Haldane's experiments this was 0·557 g., in ours 0·357 g. The loss of this quantity of alkali from the whole body could not, however, be detected by Haldane in the CO_2-absorption curve of the blood, nor by us in the titration value of the bicarbonate of the plasma.

SUMMARY.

1. Repeated observations on the hydrogen ion concentration of the blood during forced breathing have shown that the p_H rises, rapidly reaching a maximal increase of 0·20 to 0·24 in 10 to 15 minutes. When forced breathing is stopped the p_H falls even more rapidly, returning to normal within 5 minutes.

2. Simultaneous observations on the p_H and the bicarbonate of the plasma and on the alveolar CO_2 under normal conditions and during forced breathing have been made, and show that the rise in the p_H during forced breathing corresponds to the alteration found in the concentration of the bicarbonate of the plasma and the tension of CO_2 in the alveoli respectively.

We are greatly indebted to Sir C. J. Martin for much advice and helpful criticism.

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CX. THE INFLUENCE OF MEALS ON THE RISE OF THE HYDROGEN ION CONCENTRATION OF THE BLOOD DURING HYPERPNOEA.

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(*Received June 30th, 1927.*)

In the foregoing paper on the rapid rise in the p_H of the blood which occurs in forced breathing [Lepper and Martland, 1927] we confined ourselves to the discussion of experiments made not more than 2 hours after a meal. If the forced breathing was carried out 3 or 4 hours after food the course of events was always rather different, the p_H of the blood rising much more slowly and the maximum increase being never so great.

A number of experiments were made, some just before and others shortly after a meal, other conditions being as far as possible identical. The subject of the experiments (E. H. L.) had acquired considerable experience in forced breathing, and great care was taken to keep the rate and character of the respirations uniform in every case.

Fig. 1 gives the results of two typical forced breathing experiments carried out (*A*) just before, and (*B*) soon after lunch.

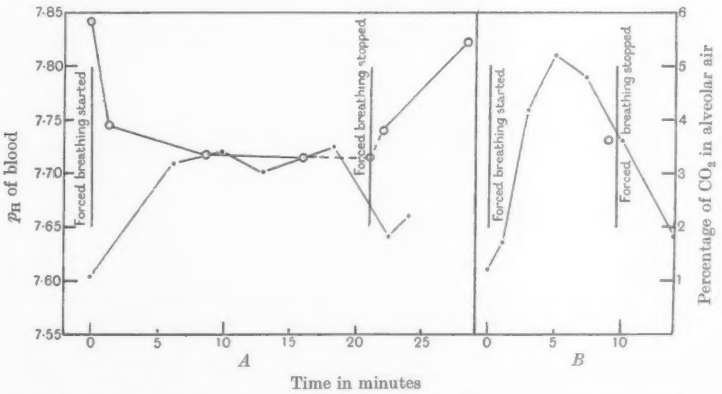

Fig. 1. The effect of forced breathing on the p_H of the blood before and after meals.

• —— • —— • —— • p_H of blood.

O——O——O——O Percentage of CO_2 in alveolar air.

A. Before meals. *B*. After meals.

All our comparable observations are set forth in Table I. The determinations were not always made at intervals of exactly 5, 10 or 15 minutes, but to simplify the tabular record of the results, graphs of each experiment were drawn from which values for these intervals were derived by interpolation. These are combined in Fig. 2 which shows composite curves of the observations on the p_H of the blood and the alveolar CO_2 made before and after meals. Table I shows that for equal ventilation the level of p_H reached is always lower before meals than after by not less than 0·09.

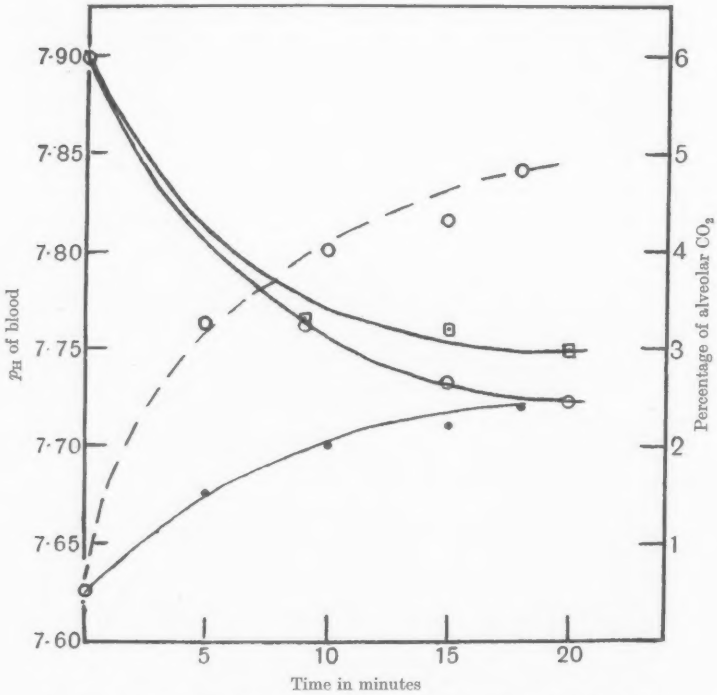

Fig. 2. Composite curves of the p_H of the blood and of the alveolar CO_2 during forced breathing experiments, carried out before and after meals

• —— • —— • —— • p_H of blood before meals.
○ — — ○ — — ○ — — ○ p_H of blood after meals.
○ —— ○ —— ○ alveolar CO_2 after meals.
⊡ —— ⊡ —— ⊡ alveolar CO_2 before meals.

The alveolar CO_2 showed a less marked fall than in the experiments carried out soon after a meal. Notwithstanding the same respiratory efforts it was not possible to reach the same low level of CO_2 in the alveolar air before as after meals. Nevertheless, this factor is not wholly responsible for the different behaviour of the p_H of the blood. Before meals the blood appears to be more

efficiently buffered. Perhaps, while gastric juice is being secreted into the stomach, hydrochloric acid escapes less readily from the corpuscles to adjust changes in the hydrogen ion concentration of the blood. This would explain why the alveolar CO_2 can be reduced to a lower level while gastric digestion is proceeding.

Table I.

Time after meal hours	mins.	Duration of forced breathing: p_H of capillary blood 0 min.	5 mins.	10 mins	15 mins.	18 mins.	Alveolar CO_2 % 0 min.	9 mins.	15 mins.	20 mins.
3	15	7·605	7·69	7·72	7·712	7·722	5·85	3·35	3·30	—
4	30	7·625	7·705	7·73	—	—	—	—	—	—
4	45	7·629	7·65	7·655	7·70	7·718	6·04	3·20	3·09	3·0
5	0	7·625	7·66	7·70	7·715	7·718	6·25	3·31	—	—
	Means	7·621	7·676	7·70	7·71	7·72	6·05	3·28	3·19	—
0	20	7·635	7·735	7·775	7·79	—	5·87	3·10	2·75	2·54
0	40	7·61	7·81	—	—	—	—	3·60	—	—
0	45	7·637	7·735	7·81	—	—	—	—	—	—
1	15	7·617	7·79	7·835	—	—	—	—	—	—
1	30	7·635	7·75	7·795	7·827	7·848	5·87	2·85	2·60	2·40
1	30	7·607	7·715	7·80	7·83	7·835	6·05	—	—	2·48
	Means	7·624	7·763	7·80	7·816	7·84	5·93	3·18	2·65	2·46

SUMMARY.

In forced breathing experiments carried out 3 or more hours after meals, the p_H of the blood rises more slowly, and the maximum change is less, than in similar experiments performed within 2 hours after food. Under the former conditions, the alveolar CO_2 cannot be reduced to the same extent by the same rate and depth of respiration.

We are greatly indebted to Sir C. J. Martin for much helpful advice and criticism.

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CXI. A NOTE ON THE TEMPORARY SPONTANEOUS DISAPPEARANCE OF TYPICAL "BERI-BERI" SYMPTOMS IN PIGEONS FED ON DIETS DEFICIENT IN VITAMIN B.

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(*Received June 20th, 1927.*)

It has been previously reported [Kon and Drummond, 1927] that temporary improvement of the typical nervous symptoms was observed in several "beriberi" pigeons fed on a synthetic vitamin B-free diet of the type described by Randoin and Simonnet [1924]. In view of the importance of that observation it seemed necessary to repeat those experiments on a larger number of birds. Nineteen birds were actually kept under observation. The preparation of the diet and the general care of the animals were as already described [Kon and Drummond, 1927—lot 1], with the exception that the food intake was not determined; the birds were, however, weighed three times a week. The experiment lasted 54 days, during which time 4 birds (21 %), Nos. 1, 2, 5 and 7, died without showing any characteristic acute symptoms of vitamin B-deficiency. The remaining 15 (79 %) developed unmistakeable "beriberi" symptoms in the course of 24, 39, 17, 31, 34, 20, 31, 24, 28, 17, 37, 21, 47, 38 and 28 days (pigeons Nos. 3, 4, 6, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 and 20 respectively), the average period being 29 days. Of these birds 6 (32 %) (Nos. 4, 10, 11, 12, 14, 16) died without improving, whilst obvious spontaneous cures occurred in the remaining 9 (47 %), thus confirming the previous findings.

Owing to the demand on space it is not possible to give here a detailed history of each bird, nor to tabulate the results in a concise and clear table. The histories of three typical spontaneous cures are given below. Subsidence of the symptoms for a period of less than 48 hours was not considered a cure.

Pigeon No. 3. *Initial weight* 462 *g.*

Day of experiment	Weight (g.)	Temperature 0° C.	Remarks
24	305	38·5	Head retraction, convulsions
25	—	—	No symptoms
26	277	38·9	Acute symptoms provoked
27	—	39·8	Better, weak, no symptoms
28	267	41·0	" "
29	—	38·6	Looks normal, eats, but picks up food with difficulty
30	280	39·4	Crop full
31	—	38·5	Acute symptoms provoked
32	—	—	Death in convulsions

Pigeon No. 6. *Initial weight* 375 *g.*

Day of experiment	Weight (g.)	Temperature 0° C.	Remarks
17	—	37·5	Emprosthotonos, convulsions, cart-wheel turning
18	265	38·0	" " "
19	—	38·6	Much better
20	245	39·0	Looks quite normal, walks easily in the cage
21	—	40·2	" " "
22	237	38·1	" " "
23	—	—	" " "
24	222	39·8	" " "
25	—	—	" " "
26	257	40·1	Ate, can fly in the laboratory
27	—	40·1	Looks quite normal
28	228	39·9	" "
29	—	38·0	" "
30	209	37·4	Very marked opisthotonos, wasted
31	—	—	Found dead in the morning

Pigeon No. 9. *Initial weight* 445 *g.*

31	—	—	Marked head retraction, convulsions
32	266	39·0	No symptoms, better
33	—	39·0	" "
34	247	37·8	" "
35	—	39·7	Eats greedily
36	298	40·5	Crop full
37	—	39·5	No symptoms
38	292	—	"
39	—	38·1	Convulsions, partially paralysed
40	265	37·8	Very acute symptoms
41	—	38·5	Very acute symptoms, impaired respiration, used for another experiment in moribund condition

It was found impossible to foretell whether the symptoms would disappear spontaneously or end in the death of the animal, as sometimes even very severe initial symptoms lasting for 2–3 days eventually cleared and the birds recovered for a certain period of time (pigeons Nos. 15, 18), whereas in other cases the death of the animal suddenly followed the onset of the nervous disorder (pigeons Nos. 4, 12, 14, 16). Possibly, though not always, the fall of temperature accompanying the acute stage was more pronounced in those cases which did not recover (pigeons Nos. 11, 14, 16). It is very interesting that a second spontaneous cure was never observed, the birds either dying without marked symptoms some time after a first cure, or succumbing in a second attack, which without exception proved fatal. Two pigeons (Nos. 9 and 20) were used, after developing for the second time acute symptoms, for another experiment before death supervened; they were, however, already in a moribund condition. The second symptoms were as a rule of the most severe type, often associated with impaired respiration.

As regards the body temperature, it will be seen that in general the onset of acute symptoms is accompanied by a marked drop and that there is a corresponding increase when a spontaneous cure is manifested. There are, however, a few exceptions, *e.g.* pigeon 17. The body temperature of a "deficient" bird is the resultant of so many factors: inanition, impaired temperature regulation, daily fluctuations and last, but not least, infection [McCarrison,

1921], that it is almost impossible to account satisfactorily for all of them and gain a clear picture of the true condition of the animal.

In order to be sure that the spontaneous cures were not caused by an unknown disturbing factor present in the laboratory, a batch of 24 pigeons purchased at the same time and from the same source as the first 19 birds, was placed under observation simultaneously with them and under identical conditions except that their diet consisted of polished rice instead of the synthetic mixture. Those birds behaved absolutely normally: a large percentage (over 60 %) developed typical symptoms, but not a single spontaneous cure could be observed, the animals dying mostly in the course of 24, maximally 48, hours after the onset of the nervous disorder.

Whether the known deficiencies of polished rice—insufficient amount of salts and lack of roughage—are to be looked upon as the causative agents of the onset of immediately fatal "beriberi" symptoms or whether still another factor plays here a rôle can be decided only on the results of further investigations.

These experiments clearly demonstrate that a curative test, when pigeons fed on synthetic rations of the type here described are used, might lead to completely erroneous conclusions.

SUMMARY.

The occurrence of temporary spontaneous cures in a large percentage of pigeons fed on a vitamin B-deficient but otherwise complete synthetic ration of the type described by Randoin and Simonnet reported in a previous communication of Kon and Drummond is fully confirmed. Pigeons fed on synthetic rations should not be used for the curative test.

I wish to take this opportunity of expressing my deep gratitude to Prof. J. C. Drummond for the hospitality of his laboratory and for other facilities which he so kindly extended to me, and also to thank the International Health Board of the Rockefeller Foundation for a Fellowship, during the tenure of which this work was carried out. The expenses were defrayed from a grant made by the Medical Research Council.

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CXII. THE EFFECT OF THE ADMINISTRATION OF GLYCINE TO PIGEONS ON A DIET DEFICIENT IN VITAMIN B.

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(*Received June 23rd, 1927.*)

It is believed by various authorities [see Funk, 1914; Funk and Collazo, 1925; Randoin and Simonnet, 1924, 1, 2] that protein metabolism requires less vitamin B than that of carbohydrate. In fact these authors consider vitamin B as specially associated with the utilisation of carbohydrates, the last-named authors even believing that experimental animals (*e.g.* pigeons and rats) can be kept alive in a good state of nutrition on dietaries deprived both of vitamin B and of carbohydrates. Such theories have been fully dealt with in a previous communication [Kon and Drummond, 1927], in which observations were reported in disagreement with them. It seemed worth while, however, to ascertain whether the alleged sparing action attributed to proteins could be explained by reference to their specific dynamic action, leading to increased cell metabolism and possibly to liberation of the body reserves of vitamin B. In order to investigate this question, it was decided to administer to pigeons fed on a diet deficient in vitamin B sufficient amounts of glycine to raise markedly their metabolism. Three lots of pigeons, each consisting of four birds, were used. The initial average weights were 428, 421 and 411 g. respectively. At first they were fed on polished rice for 12 days until the typical decrease in appetite was manifested, by which time the average weights were 393, 388 and 362 g. respectively. The actual experiment was then started, the first lot receiving daily a solution of 2 g. of pure synthetic glycine in 25 cc. of water, the second a solution of 1·6 g. of glucose in 12 cc. of water (glycine gives rise to 80 % of its weight of glucose [Ringer and Lusk, 1910]), the third 25 cc. of water. Ample supplies of polished rice and water were also provided for each group. All the liquids were introduced into the crop by means of a small funnel and a piece of rubber tubing. The weight of the birds, their food intake and cloacal temperature were determined daily. The glycine and glucose solutions were diluted as far as convenient for administration in order to avoid the introduction of strongly hypertonic fluids. Nevertheless, the glucose solution had roughly 2·4 times the osmotic pressure of blood, and the glycine 3·3. Vomiting was observed in all the pigeons of the glycine group. It was not present in the two remaining sets. Vomiting was frequently observed by Csonka [1915] after administering glycine to dogs.

Effects of dosage. The birds receiving glycine died in the course of 30, 24, 21 and 25 days respectively (on the 18th, 12th, 9th and 13th days after the administration of glycine was commenced) without showing any characteristic symptoms other than loss of appetite, weakness and in two cases paralysis. Nothing of interest was revealed on *post mortem* examination with the exception of a marked enteritis in one case (pigeon 3). One of the pigeons receiving sugar developed opisthotonos on the 28th day of the experiment, one died without marked symptoms on the 27th day. The experiment was terminated on the 31st day, after all the pigeons receiving glycine had died. Two of the sugar group and all the birds of the water group were still surviving at that time.

Body weight. After 8 days of actual experiment (up to the death of the first pigeon of the glycine group) the average weights were 339, 339 and 321 g. respectively, the pigeons on glycine having lost 54 g., or an average of 6·8 g. daily, those on sugar 49 g., or an average of 6·1 g. per day, and the birds receiving water 41 g., or 5·1 g. daily.

Food intake. The average daily food intake, that is, the voluntary consumption of polished rice, for the same period of time was 0·5 g. in the glycine group, 1·6 g. in the sugar group and 4·2 g. in the water group. It appeared that administration of glycine and glucose interfered with the voluntary food intake, in the former case more strongly than the latter.

Body temperature. The average temperatures at the beginning of the actual experimental period were 41·7°, 41·9° and 41·8°; at the end of the experiment these had dropped to 39·6°, 40·7° and 40·4°, the heaviest drop being manifested by the glycine group.

It is evident that the administration of a substance having a marked specific dynamic action had no beneficial effect on pigeons deprived of vitamin B and that on the contrary these birds declined rapidly. There is little doubt that glycine had no toxic action *per se*, and that probably the bad effects were caused by the high molecular concentration of the liquid administered. The molecular concentration of the glucose was lower and hence the untoward results were much less marked. It will be remembered that in pigeons forcibly fed deficient diets, the crop does not function normally [Kon and Drummond, 1927], and that the hypertonic fluid consequently could not be disposed of so easily as in normal animals.

Funk and Paton [1922] observed occlusion of the crop and death in pigeons fed forcibly a mixture of glucose and egg-albumin, and attributed these symptoms to a difference in osmotic pressure of the sugar solution and the body fluid. Collazo [1923] introduced large amounts (10 g.) of various carbohydrates into the crops of vitamin B-deficient and normal pigeons and found that beriberi symptoms and death, or death without previous symptoms, ensued invariably in the former. Glucose, galactose and fructose showed the highest, and starch the lowest toxicity, whereas the disaccharides sucrose, lactose and maltose, occupied an intermediate position. No symptoms were

produced in normal animals even by larger doses. Collazo [1923] concludes from his experiments that in vitamin B avitaminosis, the carbohydrate metabolism becomes aberrant and toxic intermediate substances are produced. It seems that a much simpler explanation is that, the emptying of the crop being impaired in vitamin B-deficient pigeons, they were killed by the enormous osmotic pressure of the solutions. Collazo does not give the concentration but it was surely not less than 20 % as even the administration at once of 50 cc. of liquid would present difficulties[1]. In normal pigeons the rapid emptying of the crop not only exposes the hypertonic liquid to the large surface of the gut and thus ensures prompt dilution and excretion, but also enables the birds to drink water. The supposition is further strengthened by the fact that the toxicity of the substances administered was inversely proportional to their molecular weight (starch, disaccharides, monosaccharides). It is to be noted that in the dog administration of glucose (100 g.) never caused death, but vomiting, a symptom observed by Cowgill [1921] even when vitamin B-containing extracts were given by the stomach tube to vitamin B-deficient dogs. Collazo also found that administration of 1 g. glucose daily to a pigeon, previously kept for 1 week on a vitamin B-deficient ration, caused convulsions and death in the course of 3 days. As already reported, out of four pigeons receiving nearly twice this dose, two died 12 and 16 days respectively after the administration was commenced. Two were still surviving after 31 days of vitamin B-free feeding and 19 days of sugar administration.

SUMMARY.

Administration of glycine has no beneficial effects on pigeons fed on diets deficient in vitamin B. The opinion of Collazo that the carbohydrates administered to vitamin B-deficient pigeons are toxic *per se* is not corroborated. The effects are attributed to the high osmotic pressure of the solutions administered and to the inability of the deficient birds to empty their crops normally.

My best thanks are due to Prof. J. C. Drummond for his interest and support during the course of the work and also to the International Health Board of the Rockefeller Foundation for a Fellowship, during the tenure of which this work was carried out.

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[1] It would appear from a subsequent publication of Rubino and Collazo [1923] that the carbohydrates were administered as 50 % solutions.

CXIII. THE EFFECT OF CYANIDE ON THE SCHARDINGER ENZYME.

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(*Received June 25th, 1927.*)

Szent-Györgyi [1926] claims to have brought forward evidence that in the aerobic oxidation of aldehydes under the influence of the aldehyde oxidase of milk (Schardinger's enzyme) an activation of the molecular oxygen by iron in accordance with Warburg's theory is necessary. His evidence consists in the observation that cyanide in a concentration of $M/200$ produced a 75 % inhibition of the uptake of oxygen by the enzyme-aldehyde system, and he concludes that the cyanide acts by inactivating the iron, as it does in the cases of iron catalysis studied by Warburg and others. The question is of importance in connection with Warburg's theory of tissue oxidations, and in view of the facts that Dixon and Thurlow [1925] have shown that the xanthine oxidase system is independent of such activation by iron, and have further shown [1924, 2] that the xanthine oxidase is identical with, or at any rate very closely similar to, the Schardinger enzyme, it was clear that a more detailed study of the action of cyanides on the latter enzyme should be made.

The experiments were carried out in a Barcroft apparatus at 40°. The enzyme solution used was a 20 % solution in phosphate buffer of the caseinogen preparation of Dixon and Thurlow [1924, 1]. This preparation is very similar to that used by Szent-Györgyi, differing only in the method of precipitation. The acetaldehyde was freshly prepared from pure crystalline aldehyde-ammonia by distillation with dilute sulphuric acid. It was made up in solution in water, and at Dr Szent-Györgyi's suggestion the solution was made alkaline to phenolphthalein with NaOH, allowed to stand a few moments, and re-neutralised immediately before use in order to destroy any traces of peracids which might have been formed and which might interfere with the action of the cyanide. The same amount of aldehyde solution was placed in both flasks of the apparatus in order to compensate the vapour pressure. The cyanide solution was always a freshly made up and neutralised solution of pure crystalline potassium cyanide.

Several series of experiments were carried out, and similar results were obtained in all. A typical set of curves is given in Fig. 1.

It will be seen that $M/100$ cyanide produces some inhibition of the oxygen uptake, but much less than that observed by Szent-Györgyi with $M/200$,

possibly owing to slight differences in the experimental conditions. With $M/1000$ cyanide, however, no inhibition is produced, which shows that the system is *not* cyanide-sensitive in Warburg's sense. Even with $M/300$ cyanide no effect can be observed, as Fig. 2 shows, and the inhibition only begins to be noticeable with about $M/200$.

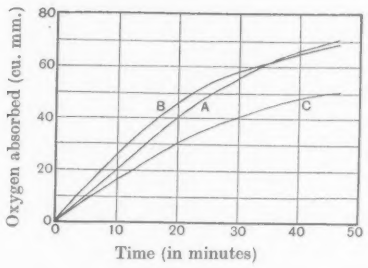

Fig. 1.

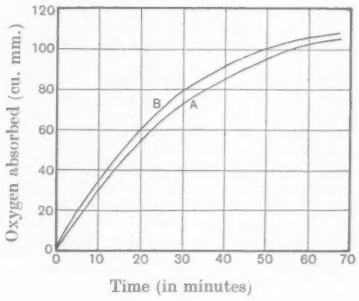

Fig. 2.

Fig 1. Each apparatus contained: 1 cc. 20 % caseinogen enzyme preparation; 1 cc. phosphate buffer (p_H 7·6); 1 cc. 0·5 % acetaldehyde; and the following concentrations of KCN: *A*, 0; *B*, $M/1000$; *C*, $M/100$. The control flasks contained the same solutions, but with buffer instead of the enzyme solution.

Fig. 2. As in the previous experiment, but a different enzyme preparation was used, and the concentrations of KCN were: *A*, 0; *B*, $M/300$.

We may compare the behaviour of this system with that of a typical cyanide-sensitive system—the succinoxidase of muscle, which was studied by Fleisch [1924]. Fleisch showed that in this case a concentration of cyanide of less than $M/1000$ was amply sufficient to produce a practically complete inhibition of the oxygen uptake in the presence of succinate. The enzyme was not, however, destroyed by the cyanide, as the methylene blue reduction was unaffected.

In order to determine whether the inhibiting action of $M/100$ cyanide on the Schardinger enzyme was due to actual destruction of the enzyme, the solutions used in the experiment given in Fig. 1 were transferred at the conclusion of the experiment from the flasks of the Barcroft apparatus to Thunberg vacuum-tubes. 1 cc. of 1/5000 methylene blue was added to each tube and the tubes were then evacuated and placed in the thermostat at 32°. There was no need to add fresh aldehyde, as only about 10 % of that originally added had been oxidised during the course of the experiment. The results obtained were as follows:

Contents of tube	Reduction-time min.
Solution from apparatus with no KCN...	98
" " $M/1000$ KCN	90
" " $M/100$ KCN	2000
1 cc. original solution + 1·1 cc. buffer + 0·9 cc. aldehyde	9

These figures show quite definitely that the inhibiting effect of the higher concentration of cyanide is due to an actual destruction of the enzyme itself. In all three solutions there had been considerable destruction during the oxygen uptake, which was doubtless due to the peroxide formed during the reaction, as already observed during the oxidation of hypoxanthine [Dixon, 1925; see also Thurlow, 1925]. The much greater destructive effect of the $M/100$ cyanide is however evident.

A precisely similar inhibitory effect of cyanide in concentrations greater than about $M/150$ was observed by Dixon and Thurlow [1925] using hypoxanthine as substrate, and shown by them to be due to the same cause.

Some unpublished experiments made by the writer and Miss Thurlow in 1924 may be given here, as they show clearly the destructive effect of cyanide on the milk oxidase, with either aldehyde or hypoxanthine as substrate. The experiments were carried out in Thunberg tubes in the usual way. The enzyme solution used was one of the caseinogen enzyme preparation in phosphate buffer at p_H 7·6.

Contents of tubes	Reduction-time min.
5 cc. enzyme + 0·5 cc. methylene blue + 0·1 cc. (0·2 mg.) hypoxanthine	5
5 cc. enzyme + 0·5 cc. methylene blue + 0·1 cc. (0·2 mg.) hypoxanthine + 0·1 cc. $M/10$ KCN	4·5
5 cc. enzyme + 0·5 cc. methylene blue + 0·1 cc. (0·2 mg.) hypoxanthine + 0·5 cc. $M/10$ KCN	7
5 cc. enzyme + 0·5 cc. methylene blue + 0·1 cc. 10 % aldehyde	7
5 cc. enzyme + 0·5 cc. methylene blue + 0·1 cc. 10 % aldehyde + 0·1 cc. $M/10$ KCN	8·5
5 cc. enzyme + 0·5 cc. methylene blue + 0·1 cc. 10 % aldehyde + 0·5 cc. $M/10$ KCN	17

These figures show that while $M/500$ cyanide produces no appreciable effect, $M/100$ cyanide has a definite destructive action on the enzyme; which is in good agreement with the experiments given above. The effect appears to be more marked with aldehyde than with hypoxanthine, but this does not necessarily mean that we are dealing with two distinct enzymes, as it is possible that there may also be an action of the cyanide on the aldehyde at the enzyme surface.

The destruction may become very marked if the oxidase is allowed to stand for a short time with the cyanide before adding the aldehyde and methylene blue, as the following experiment shows. 1 cc. of 20 % caseinogen preparation + 1 cc. $M/30$ KCN were allowed to stand 5 minutes at room temperature, and then 1 cc. 1 % aldehyde + 1 cc. buffer and 1 cc. methylene blue added. The reduction-time was 30 minutes, whereas 1 cc. of the same enzyme solution + 1 cc. buffer + 1 cc. aldehyde + 1 cc. methylene blue reduced in $4\frac{1}{2}$ minutes.

The experiments described in this paper show clearly that the inhibition produced by the higher concentrations of cyanide cannot be regarded as evidence for oxygen-activation. Since it occurs with methylene blue as the hydrogen acceptor under precisely the same conditions as with oxygen it must be referred rather to an actual destruction of the enzyme.

SUMMARY.

Inhibition of the aerobic oxidation of aldehyde by the Schardinger enzyme is produced only by concentrations of cyanide greater than $M/200$. It is due to an actual destruction of the enzyme itself, and does not constitute evidence of oxygen-activation.

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CXIV. ON THE REDUCING POWER OF GLUTATHIONE AND CYSTEINE.

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(*Received June 25th, 1927.*)

Kendall and Nord [1926] in a recent paper have put forward a somewhat novel view of the mechanism of reduction by —SH groups. They state that, in the absence of oxygen, cysteine and reduced glutathione are unable to reduce indigo carmine, but that in the presence of small amounts of oxygen an active addition compound of the —SH group with oxygen is formed, whereby the system acquires the power to reduce the dye. The same "activation" was obtained by the addition of hydrogen peroxide or of sodium disulphide.

In view of the importance of such a fact in considering the functions of sulphur compounds in cell respiration processes, the experimental basis for such a view calls for careful consideration.

The reducing power of cysteine and glutathione in the absence of oxygen.

Kendall and Nord placed solutions of indigo carmine and cysteine in phosphate buffer in a vessel through which nitrogen was bubbled continuously. After 40 hours at 30° the solutions still remained deep blue, from which they concluded that no reduction of the dye had taken place. This result was at variance with some unpublished observations made by us in 1922, in which, using the Thunberg vacuum-tube technique, rapid reduction of indigo carmine by cysteine was found to occur. It seemed to us that the discrepancy might be due to the presence of traces of oxygen, particularly in view of experience of the difficulty in obtaining nitrogen in quantity free from such traces.

In view of the large volume of gas which Kendall and Nord must have passed through their solution it will be clear that a very small percentage of oxygen in the nitrogen might have been sufficient to prevent decoloration, owing to the great ease with which reduced indigo carmine is reoxidised by oxygen. Assuming (information on this point being absent from Kendall and Nord's paper) a rate of bubbling of 1 cc. per second, then in 40 hours 144 litres of nitrogen would flow. Even if the gas were 99·99 % pure this would mean that nearly 15 cc. of oxygen would pass into the solution during the experiment, a quantity sufficient to reoxidise the whole of the indigo carmine many times over.

In order to decide whether cysteine can reduce indigo carmine in the absence of oxygen we made experiments, taking extreme precautions to remove all traces of oxygen. For this purpose the special vacuum-tube represented in Fig. 1 in front and side view was employed.

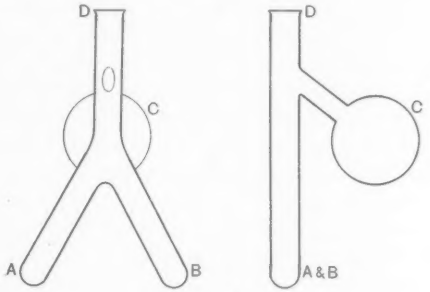

Fig. 1.

The open end *D* was provided with a well-fitting rubber stopper carrying a glass tube with a tap, so that the tube could be evacuated. The neutral cysteine solution (previously kept in an exhausted vacuum-tube for 2 or 3 days) was placed with an appropriate volume of buffer solution (p_H 7·6) in the limb *A*, and the indigo carmine solution in *B*. A strong solution of sodium hydrosulphite in water and caustic soda were placed in the bulb *C* by means of a special curved pipette, and the apparatus immediately exhausted by an efficient water-pump. When no further bubbles of dissolved gas could be obtained from the liquids, nitrogen was admitted and the apparatus re-exhausted. The process was again repeated, the tap closed after the final exhaustion and the apparatus allowed to stand for 24 hours or more with gentle shaking at intervals. At the end of this period the solutions in *A* and *B* were carefully mixed by tilting the tube, and the apparatus was placed in the bath at 40°. (The apparatus was so constructed and handled that there was no possibility of any trace of the hydrosulphite getting into the reacting solutions. In no case have we observed the slightest reduction of the dye before the solutions were mixed, or in control experiments without the —SH compound.) In all cases rapid and complete reduction of the indigo carmine occurred. Using 0·3 cc. indigo carmine (0·035 *M*) + 1·0 cc. cysteine (0·05 *M*) + 3 cc. phosphate buffer the dye was completely reduced in 6 minutes. At the end of the experiment the vacuum in the tube was tested by immersing the tube in water and opening the tap. The water filled the tube with the exception of a small bubble of approximately 50 mm.^{3}. Assuming that our nitrogen contained as much as 1 % of oxygen it will be clear that the maximum possible quantity of oxygen in the tube at the end of the final exhaustion is 0·5 mm.^{3}, and this will of course be rapidly and completely absorbed by the hydrosulphite solution, which as is well known is an extremely efficient deoxygenating agent.

In a series of experiments we found that the more carefully traces of oxygen are removed, the smaller becomes the time for reduction of the dye (provided that large amounts of oxygen are not present; cf. the following section).

Similar results have been obtained with reduced glutathione.

The preparations of cysteine used by us, though very pure, were not absolutely iron-free in Warburg's sense, although the fact that no trace of colour developed in the solutions on making faintly alkaline with ammonia and shaking with oxygen shows that this was very nearly the case. Kendall and Nord freed their cysteine preparations from iron by the Warburg and Sakuma [1923] technique; but the effect of this was largely negatived by the fact that they used, without purification, commercial indigo carmine, which would certainly contain small amounts of iron. In view of the recent work of Harrison [1927], who found that the reduction of methylene blue by cysteine was largely (but not entirely) due to catalysis by traces of iron, it became necessary to consider how far the reduction of indigo carmine observed by us was due to the same cause. We obtained with indigo carmine results very similar to those obtained by Harrison with methylene blue. The addition of very small traces of ferric chloride produced a marked acceleration of the reduction, while the addition of a small amount of KCN slowed the reaction considerably. But, as is the case with methylene blue, even when the metal-catalysis is completely abolished by the addition of KCN, and when all traces of oxygen are removed, the reaction still proceeds with a quite considerable velocity.

We consider that these results dispose of Kendall and Nord's contention that cysteine and glutathione cannot reduce indigo carmine in the absence of oxygen. As this contention is fundamental to their theory, we propose to discuss the bearing of the facts given above on their evidence for the formation of an oxygen addition compound.

When Kendall and Nord stopped the flow of gas through their solutions, and allowed them to stand undisturbed for some hours, they observed that occasionally the dye became reduced, with the exception of a blue layer on the surface of the liquid. Kendall and Nord concluded that in these cases oxygen had leaked into the apparatus, and by its presence had enabled the reduction to be brought about, owing to some activating property. In order to test this hypothesis they proceeded as follows. After bubbling nitrogen through the mixture of cysteine and indigo carmine for several hours, with no apparent reduction of the dye, the bubbling was stopped and 10 cc. of air admitted. On allowing to stand undisturbed for 2 hours, reduction was complete in a layer 2 cm. below the surface.

A possible interpretation of this experiment seems to be that the traces of oxygen continuously introduced by the nitrogen maintained the dye in its oxidised form. On cessation of bubbling the rate of diffusion of oxygen into the deeper layers of the solution is not sufficient to mask the effect of the reduction by the —SH compound. There therefore results a colourless deep layer and a blue surface layer as Kendall and Nord observed.

This conclusion is strikingly supported by the following internal evidence in Kendall and Nord's account.

(1) "The reduction was retarded in proportion to the agitation," *i.e.* to the volume of gas passing through the solution.

(2) "When the air was mixed with the nitrogen and bubbled through the solution, little or no reduction of the indigo carmine occurred"; this is a very significant statement, since there seems no reason on their view why oxygen leaking into the apparatus should be able to cause the formation of the addition product, while oxygen deliberately introduced with the nitrogen should be unable to act. In order to account for this result Kendall and Nord were obliged to assume that agitation prevents the formation of the oxygen addition product!

In view of the above facts, which would appear to invalidate Kendall and Nord's experiments, we considered it necessary to reinvestigate the question whether oxygen can *accelerate* the reduction of indigo carmine. In the presence of fairly large amounts of oxygen we have been able to observe such an acceleration. For instance, if a neutral solution containing cysteine and indigo carmine is divided into two portions, one being placed in an evacuated Thunberg tube and the other in an open test-tube, it will be found that reduction is complete in the open tube (except of course at the surface of the liquid) definitely sooner than in the vacuum-tube.

We are of the opinion that this phenomenon is due to the hydrogen peroxide formed during the autoxidation of the —SH group [Thurlow, 1925], and we discuss its interpretation more fully in the following section of the paper.

The rôle of hydrogen peroxide in the formation of the addition compound.

Kendall and Nord state that hydrogen dioxide is also able to form an active addition compound: "hydrogen dioxide was therefore added to solutions of cysteine and after some time indigo carmine added. No reduction occurred. The indigo carmine was then added to the cysteine solution before the hydrogen dioxide. With the conditions thus produced the indigo carmine was reduced almost immediately providing sufficient hydrogen dioxide had been used." Kendall and Nord consider that these results indicate the existence of an essential intermediate compound which is formed by the action of hydrogen dioxide on cysteine.

We have confirmed these observations, but we do not agree with this interpretation of the phenomenon. It is obvious that the action of the hydrogen peroxide may be either on the cysteine or on the indigo carmine. The fact that according to Kendall and Nord the oxygen addition compound is only formed when indigo carmine is present, for which they offer no satisfactory explanation, suggests that the active compound may be due to the action of the peroxide on the dye rather than on the cysteine. It is well known that hydrogen per-

oxide reacts readily with indigo carmine, and with dyes on which hydrogen peroxide has no action Kendall and Nord failed to obtain effects comparable with those observed with indigo carmine. We have found for instance that hydrogen peroxide produces no acceleration of the reduction of methylene blue by cysteine.

In order to test the possibility that a catalytically active compound is formed from the indigo carmine we carried out the following experiments.

A solution of indigo carmine in phosphate buffer p_H 7·6 was treated with dilute hydrogen peroxide, and the mixture allowed to react until *slight* bleaching had occurred. Manganese dioxide was then added, the solution warmed to decompose all traces of H_2O_2, and the mixture filtered (solution *A*). A second solution of untreated indigo carmine was then prepared (solution *B*) of the same depth of colour as solution *A*. The two solutions were thus identical, except that *A* contained a small amount of the oxidation-product of indigo carmine. Equal volumes of the two solutions were placed separately in two Thunberg tubes, equal amounts of cysteine solution added to each, and both tubes thoroughly evacuated. It was found that the treated indigo carmine solution (solution *A*) was reduced with very great rapidity compared with solution *B*, the acceleration produced being comparable with that produced by the direct addition of hydrogen peroxide. That a true reduction, and not merely a bleaching, had occurred was shown by the ready reappearance of the colour on the admission of oxygen. A control was carried out, to make certain that all traces of peroxide had been removed, by adding manganese dioxide to dilute hydrogen peroxide of the same strength as previously and treating in exactly the same way as before. The filtrate gave no test with KI and starch, and on addition to a mixture of indigo carmine and cysteine no accelerating effect could be observed. A further control, in which indigo carmine was treated with MnO_2 without H_2O_2, gave no acceleration; showing that the acceleration previously observed was not due to catalytic action by traces of manganese.

The result described shows conclusively that by the action of hydrogen peroxide on indigo carmine products are formed which have a marked catalytic effect on the reduction of indigo carmine by cysteine. We consider that this fact provides the true explanation of the phenomena observed by Kendall and Nord, and renders unnecessary the assumption of the formation of an active oxygen addition product with cysteine.

The fact that no effect was observed by Kendall and Nord when the hydrogen peroxide was added to the cysteine, and indigo carmine added only after some time, is clearly due to the removal of the H_2O_2 by the usual reaction with the —SH group before the addition of the indigo carmine. By reduction-potential measurements we have found that under the above conditions, removal of the peroxide is complete in a few minutes.

Hydrogen peroxide (as is so frequently the case) probably requires traces of iron as a catalyst for its action on the indigo carmine. We have found that

minute traces of ferric or ferrous salts have a marked accelerating effect on the bleaching of indigo carmine by H_2O_2; and we have shown that the products of the reaction are active accelerators. Moreover, we have found that cyanide definitely inhibits this reaction. Now if our interpretation be the correct one we should expect to find that the addition of a small amount of KCN, by preventing the action of the peroxide on the dye, would abolish the accelerating effect of the H_2O_2. This is precisely what occurs, as the following experiment shows.

Contents of tubes	Reduction-time at 35°
Indigo carmine + buffer + cysteine	1 hr.
" + " + " + H_2O_2	17 mins.
" + " + " + " + KCN	3 hrs.
" + " + " + KCN	4 hrs.

The quantities used were:

Indigo carmine: 1 cc. of 0·035 *M*.
Cysteine: 1·5 cc. of 0·1 *M*.
H_2O_2: 0·5 cc. of 0·1 *M*.
KCN: final concentration 0·005 *M*.
Phosphate buffer: 3 cc. $p_H = 7·6$.

It will be seen that the presence of the cyanide completely prevents the acceleration by the peroxide, although the normal reduction of the indigo carmine by the cysteine still occurs, as mentioned above.

We conclude from these facts that Kendall and Nord's main phenomenon is dependent on the presence of traces of iron present as impurities, perhaps as simple iron salts, perhaps as a cysteine-iron complex.

The rôle of sodium disulphide in the formation of the addition compound.

Kendall and Nord state that addition of 0·01 *N* sodium disulphide solution to cysteine in the presence of indigo carmine brought about the prompt reduction of the dye, and they claim that "cysteine reacts with sodium disulphide in the same manner as it does with hydrogen dioxide."

No information is given as to the method of preparation of the disulphide solution. As ordinarily prepared, by dissolving the theoretical amount of sulphur in a solution of Na_2S, the solution is a most powerful reducing agent, and we have found that it reduces indigo carmine with remarkable rapidity. Kendall and Nord, in fact, also observed this reducing power.

It therefore seems, to say the least, inadvisable to draw such a definite conclusion as that of Kendall and Nord, since the reduction of the dye may be due to the sodium disulphide and not to the cysteine. Kendall and Nord do not appear to have carried out control experiments with sodium disulphide alone.

Reduction-potential measurements.

Dixon and Quastel [1923] pointed out the unsuitability of the platinum electrode for work on the —SH system, owing to the extensive potential drifts which are met with using this electrode. Kendall and Nord nevertheless carried out all their work with this electrode. They "obtained systems in which the drifts had been eliminated" by simply waiting until the drifts had ceased, and taking the final reading. The significance of these values seems somewhat doubtful.

Furthermore, the hydrogen ion concentrations of Kendall and Nord's solutions were not accurately controlled, and moreover were undetermined. The additions made at various times to their electrode vessels consisted of such solutions as those of cysteine hydrochloride, the sodium salt of cystine, sodium disulphide, and un-neutralised glutathione, together with "sufficient $N/10$ sodium hydroxide to neutralise *all or part* of the acidity" (our italics).

Their method of preparation of solutions of reduced indigo, which they used in experiments on the reversibility of the —SH, —SS— system, seems also to call for some notice [see Kendall and Ort, 1926]. The indigo was reduced by boiling with glucose and sodium hydroxide; and the whole of the resulting mixture of reduced indigo, alkali, and the decomposition products of glucose was used for addition to the electrode vessel, and simply referred to as "reduced indigo" by Kendall and Nord. It will be obvious that such a solution, containing one of the most powerful reducing agents—glucose in the presence of alkali—together with the unknown decomposition products of glucose, will give reduction-potential readings of little significance.

Kendall and Nord state that neutralisation of the above solution was unnecessary, as on oxidising the reduced indigo and filtering off the indigo the resulting solution was neutral to phenol red. We have found, however, that this indicator cannot be used to determine the p_H of such a solution, as on addition of phenol red to a solution of glucose and NaOH which has been boiled, the indicator always assumes a colour approximately corresponding to p_H 7·2. Addition of 40 % caustic soda fails to alter this colour, so that this indicator gives no information as to the p_H of such solutions.

In order to ascertain as far as possible the actual p_H of the solution used by Kendall and Nord we prepared a solution of reduced indigo by their procedure, using the exact quantities given in their paper. After reoxidising and filtering off the indigo the filtrate was found to be alkaline to alizarin (which behaves normally in such solutions); and its p_H must therefore have been at least 12, although it appeared to be neutral to phenol red. With phenolphthalein the maximum red colour was first produced, but this colour vanished almost instantaneously, owing to the reducing properties of the solution.

One of the main points of Kendall and Nord's paper was that in presence of the oxygen addition compound the reduction-potential was determined by the ratio of the concentrations of the —SH compound to the —SS— com-

pound, *i.e.* there was a truly reversible system. Examination of their experimental evidence for this view fails however to establish their conclusion.

Taking a constant amount of cysteine in four electrode vessels, and oxidising varying proportions to cystine by adding different quantities of hydrogen peroxide in the presence of indigo carmine, Kendall and Nord found that the reduction-potential was highest where the ratio —SH : —SS— was highest, and varied directly with this ratio. Without further discussion they state that "it is therefore evident that the ratio of cystein to cystin establishes the equilibrium point in these solutions."

Since Dixon and Quastel found that the potential was determined solely by the concentration of the —SH compound, and that the —SS— group is without effect, it is obvious that varying the —SH concentration in the manner described by Kendall and Nord will result in variations of the reduction-potential in the sense observed by them. Any conclusion as to the importance of the *ratio* is fallacious unless the concentration of the —SH group is maintained constant and the concentration of the —SS— group varied, an experiment of which Kendall and Nord make no mention. Kendall and Nord themselves state that the —SS— form does not affect the electrode, thus showing clearly that the potential is determined solely by the —SH concentration and not by the ratio of this to the —SS—. The results obtained by Dixon and Quastel adequately explain the above observations.

Thus it appears that Kendall and Nord have brought forward no evidence of the reversibility of the —SH, —SS— system.

We do not propose to attempt any detailed discussion of the elaborate theories put forward by Kendall and Nord to account for the apparent properties of the hypothetical addition compound. We have confined ourselves in this paper to a consideration of the main points on which these theories are based. In any case it is sure that their results can have but limited application, since they refer only to the reduction of the particular dye indigo carmine, and do not occur with other dyes such as methylene blue. Still less could they have significance in tissue oxidation processes. The claim of Kendall and Nord that the active addition compound of glutathione with oxygen is the *sine qua non* for its physiological activity is not justified by the evidence.

SUMMARY.

There is no necessity for Kendall and Nord's assumption of an active addition compound of the —SH group with oxygen.

Their experiments do not show that the —SH, —SS— system is reversible.

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CXV. NOTE ON THE ERLENMEYER AMINO-ACID SYNTHESIS.

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(*Received June 27th, 1927.*)

The series of reactions, constituting a general synthesis of α-amino-acids, devised by Erlenmeyer [1893] and since employed by many other workers, can be conducted, in most cases, with little trouble and excellent yields except at one stage. The condensation of an aromatic aldehyde with hippuric acid and the conversion of the resulting azlactone into the corresponding α-benzoylaminoacrylic acid proceed almost always in yields of 70 % or over. The ease with which the acrylic acid derivative may be reduced to the corresponding propionic acid varies considerably, however, in different cases. Moreover, even in those cases in which the ultimate yield obtainable is fairly high, the reaction is apt to be somewhat troublesome to carry out. In order to bring about this reduction, Erlenmeyer employed sodium amalgam, in which he has been followed by the majority of subsequent workers, although occasionally [cf. Barger and Ewins, 1917] sodium in alcohol has been used with success.

In the synthesis of thyroxine [Harington and Barger, 1927] where it was desired to utilise Erlenmeyer's method for the preparation of β-3 : 5-diiodo-4-[4′-(hydroxyphenoxy)phenyl]-α-aminopropionic acid, the use of any alkaline reducing agent was precluded by the presence of iodine atoms in the molecule. We attempted at first to surmount the difficulty by using hydrogen and palladium in neutral or acid solution, but our efforts in this direction were entirely without success. It then occurred to us that the desired end might be achieved by the use of hydriodic acid and red phosphorus, since we had previously obtained evidence that the iodine atoms, in the compounds with which we were working, were stable towards these reagents. This experiment was successful, and we were able, by boiling the α-benzoylamino-3 : 5-diiodo-4-(4′-methoxyphenoxy)cinnamic ester with hydriodic acid and red phosphorus, to obtain a 25 % yield of the desired amino-acid.

Later it became necessary, for further work, to prepare larger quantities of this amino-acid, and experiments were therefore undertaken with the object of improving the yield. Without going into the various unsuccessful variations that were tried, it may be said at once that we have succeeded in

improving the above-mentioned yield of 25 % to one of 82 % by the simple modification of substituting for the constant boiling hydriodic acid originally employed a mixture of equal parts of the latter and of acetic anhydride. This mixture is an excellent solvent, and, by its use, the reaction could be made to proceed smoothly and to yield a clean product which could be worked up without difficulty. It may further be remarked that the whole reaction, *i.e.* the reduction and the simultaneous hydrolytic removal of the benzoyl group, was complete after boiling for 1½ hours with the hydriodic acid-acetic anhydride mixture, so that the saving of time effected, as compared with Erlenmeyer's original method, even supposing the latter to have been applicable in this case, was very considerable.

The surprising success of this method in the case under discussion, and its great rapidity, led us to investigate its applicability to the synthesis of some other amino-acids. In particular were we interested to try to prepare in this way 3 : 4-dihydroxyphenylalanine, the synthesis of which by Erlenmeyer's method has been carried out by Funk [1911], but with unsatisfactory yields. Using vanillin as our starting point we were indeed able to prepare this amino-acid in yields which were satisfactory, whilst not being so good as those which we obtained in other cases. We have further applied the method to the synthesis of phenylalanine, tyrosine, and desiodothyroxine, the results in all cases being superior to those obtainable by Erlenmeyer's original method.

It will be noticed in the experimental part that we have employed in some instances the benzoylaminoacrylic acid and in others the ester; we have observed slight but definite differences in the yields of the different amino-acids obtainable from the acid and the ester; it appears to be a matter of experiment to determine, in any given case, which is the more suitable.

Experimental.

β-[3 : 5-Diiodo-4-(4′-hydroxyphenoxy)phenyl]-α-aminopropionic acid.

α-Benzoylamino-3 : 5-diiodo-4-(4′-methoxyphenoxy)cinnamic acid. The azlactone prepared by the condensation of 3 : 5-diiodo-4-(4′-methoxyphenoxy)-benzaldehyde with hippuric acid [cf. Harington and Barger, 1927] was dropped into 100 parts of a boiling solution of 1 % sodium hydroxide in 70 % alcohol; the substance passed rapidly into solution and the reaction was complete after boiling for 5 minutes. The hot solution was acidified with hydrochloric acid, whereupon the acid began immediately to crystallise; after standing a few hours in the ice-chest, the acid was filtered off; the first crop so obtained amounted to 60 % of the theoretical yield; a further 30 % was obtained by heating the filtrate to boiling, diluting with an equal volume of hot water, and again cooling; the total yield was therefore almost quantitative. The acid was moderately soluble in hot alcohol, and sparingly soluble in water; on recrystallisation from 90 % alcohol it formed colourless needles, M.P. 239–241° (decomp.).

Analysis. 18·3 mg. gave 0·38 mg. N (micro-Kjeldahl).
Calculated for $C_{23}H_{17}O_5NI_2$ 2·2 % N; found 2·1 % N.

β-[3 : 5-Diiodo-4-(4′-hydroxyphenoxy)phenyl]-α-aminopropionic acid. 5 g. of the foregoing acid were boiled for 1¼ hours under a reflux condenser with a mixture of 25 cc. hydriodic acid (Sp. Gr. 1·7) and 25 cc. acetic anhydride together with 3 g. red phosphorus. The solution was filtered hot through asbestos into a Claisen flask, the phosphorus being washed with acetic acid. The filtrate was evaporated to dryness *in vacuo*; some water was added and the evaporation repeated. The residue was dissolved in about 40 cc. boiling water; the solution was cooled under the tap, the flask being well shaken, and the precipitate, consisting of the hydriodide of the amino-acid mixed with benzoic acid, was filtered off and washed thoroughly with ether; the aqueous portion of the filtrate was extracted twice with ether, and warmed to remove dissolved ether. The first crop of hydriodide was added to the boiling aqueous solution, a little hydrochloric acid being added, if necessary, to obtain complete solution, and the free amino-acid precipitated by the cautious addition of ammonia; 3·35 g. of the pure amino-acid, or 82 % of the theoretical amount, were obtained.

Phenylalanine.

10 g. of α-benzoylaminocinnamic acid, obtained by the method of Erlenmeyer [1893], were boiled under a reflux condenser with 100 cc. of the hydriodic acid-acetic anhydride mixture and 10 g. of red phosphorus for 1½ hours; the solution was filtered and evaporated to dryness *in vacuo*; water was added and the evaporation repeated; the residue was shaken up with water and ether; the aqueous layer was separated, and, after a second extraction with ether, was heated to boiling and carefully neutralised with ammonia; on cooling there separated, in colourless plates, analytically pure phenylalanine. The yield was 88 % of the theory.

Analysis. 8·8 mg. gave 0·74 mg. N (micro-Kjeldahl).
14·19 mg. gave 2·0 cc. moist N_2 at 19° and 766 mm. (Van Slyke).

	Total N	NH_2-N
Calculated	8·5 %	8·5 %
Found	8·4	8·1

Tyrosine.

In a precisely similar manner there was obtained from ethyl α-benzoylamino-*p*-methoxycinnamate a 60 % yield of tyrosine.

Analysis. 15·5 mg. gave 1·2 mg. N (micro-Kjeldahl).
14·11 mg. gave 1·83 cc. moist N_2 at 19° and 766 mm. (Van Slyke).

	Total N	NH_2-N
Calculated	7·7 %	7·7 %
Found	7·7	7·4

3 : 4-*Dihydroxyphenylalanine.*

Azlactone from vanillin and hippuric acid. An intimate mixture of vanillin (15·2 g.), hippuric acid (17·9 g.), and freshly fused sodium acetate (15 g.) was treated with 30 cc. acetic anhydride and heated on the water-bath for

15 minutes. The reaction product was ground up with water, filtered, and the precipitate well washed. The crude product was crystallised from glacial acetic acid, and formed yellow needles, M.P. 189°. The yield was 75 %.

Analysis. 15·2 mg. gave 0·646 mg. N (micro-Kjeldahl).

Calculated for $C_{19}H_{15}O_5N$ 4·15 % N; found 4·25 % N.

Ethyl α-benzoylamino-3-methoxy-4-hydroxycinnamate. 10 g. of the above azlactone were dissolved in 100 cc. alcohol to which were added 10 cc. concentrated sulphuric acid, and the solution was boiled under a reflux condenser for 15–20 minutes; the greater part of the alcohol was then distilled off under reduced pressure, and the residue was poured into a dish and allowed to stand. After some days crystallisation began and, at the end of a week, was complete; the ester was filtered off and recrystallised from dilute alcohol. It formed colourless prisms, M.P. 128–129°. The yield was 65 % of the theoretical amount.

Analysis. 0·0862 g. gave 0·2109 g. CO_2; 0·0450 g. H_2O.

15·7 mg. gave 0·63 mg. N (micro-Kjeldahl).

	C	H	N
Calculated for $C_{19}H_{19}O_5N$	66·8 %	5·6 %	4·1 %
Found	66·7	5·8	4·0

It will be noted that the analysis indicates that the acetyl group, which was present in the azlactone, was split off in the process of preparation of the ester.

3 : 4-*Dihydroxyphenylalanine.* 5 g. of the ester were boiled for 1½ hours with 25 cc. hydriodic acid, 25 cc. acetic anhydride and 5 g. red phosphorus, in an atmosphere of hydrogen; the solution was filtered and evaporated to dryness under diminished pressure, hydrogen being led into the capillary tube of the Claisen flask; the aqueous solution of the residue, after removal of the benzoic acid by ether extraction, was neutralised with ammonia and evaporated to dryness under the same precautions to avoid access of air; the product was dissolved in a little water and the solution treated with a large excess of alcohol which precipitated the greater part of the pigment; the latter was rapidly filtered off and the filtrate once more taken to dryness. The residue was dissolved in hot water containing a little sulphur dioxide, the solution was boiled with charcoal and filtered, and the filtrate allowed to crystallise in a vacuum desiccator. In this way the amino-acid was obtained almost colourless and in well-formed crystals. The yield was 50 % of the theoretical. The substance melted at 269° (decomp.) and agreed in its properties with the product described by Funk [1911].

Analysis. 14·3 mg. gave 0·98 mg. N (micro-Kjeldahl).

21·3 mg. gave 2·55 cc. moist N_2 at 17° and 760 mm. (Van Slyke).

	Total N	NH_2-N
Calculated	7·1 %	7·1 %
Found	6·9	6·9

Desiodothyroxine.

The azlactone was prepared in the usual manner from 4-(4′-methoxyphenoxy)benzaldehyde [cf. Harington, 1926] and hippuric acid; there was obtained 70 % of the theoretical amount of a substance which, after crystallisation from glacial acetic acid, formed yellow needles, M.P. 141°.

Analysis. 16·0 mg. gave 0·575 mg. N (micro-Kjeldahl).

Calculated for $C_{23}H_{17}O_4N$ 3·8 % N; found 3·6 % N.

The above azlactone was converted into the acid by boiling for 5 minutes with 100 parts of 1 % sodium hydroxide in 45 % alcohol; the acid, on recrystallisation from dilute alcohol, formed colourless branched needles, M.P. 192·5°. The yield was 95 % of the theory.

Analysis. 15·8 mg. gave 0·554 mg. N (micro-Kjeldahl).

Calculated for $C_{23}H_{19}O_5N$ 3·6 % N; found 3·5 % N.

The preceding compound was converted into the amino-acid in a precisely similar manner to that described for tyrosine and phenylalanine. The yield of amino-acid was 61 % of the theoretical. The product melted at 253–254° (decomp.), and gave a hydrochloride with M.P. 239–240°; it was therefore in all respects identical with the substance previously prepared by Harington [1926].

Analysis. 19·9 mg. gave 1·02 mg. N (micro-Kjeldahl).

26·8 mg. gave 2·22 cc. moist N_2 at 17° and 760 mm. (Van Slyke).

	Total N	NH_2-N
Calculated for $C_{15}H_{15}O_4N$	5·1 %	5·1 %
Found	5·1	4·9

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CXVI. THE ENZYMIC DEAMIDATION OF ASPARAGINE IN THE HIGHER PLANTS.

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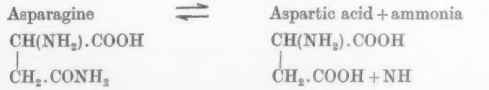
The occurrence of asparagine in the higher plant and its rôle in protein metabolism have formed the subject of numerous investigations.

It has been found that the amount normally present in the green plant is very small and it is only under artificial conditions, associated with a carbohydrate deficiency, that an accumulation of this substance is ever obtained. E. Schulze [1906], in a series of papers, showed that in etiolated seedlings most of the protein reserve is converted into asparagine. In the mature plant, in which the leaf is the seat of metabolic activity, it has been found since Schulze's work that, in general, starvation results in a conversion of protein to asparagine.

Chibnall [1924], in the course of investigations on the nitrogenous metabolism of the higher plants, concludes that there is a constant production of asparagine from protein in the normal mature leaf, and that in plant metabolism asparagine has the rôle of a translocatory substance and is the chief medium whereby nitrogen, in a form suitable for subsequent resynthesis, can be conveyed from one part of the plant to another. According to Prianischnikov [1916] asparagine is formed as a temporary nitrogen reserve whenever there occurs, through some derangement of the normal course of protein metabolism, an excess of ammonia which is toxic to the plant.

Important as is the part played by asparagine in protein metabolism, the reactions whereby protein and asparagine are interconverted are still far from being clearly understood. On the one side the degradation of protein through proteoses and polypeptides to amino-acids is well known, and a similar reverse process is probable; but there is little or no evidence regarding the steps intervening between these amino-acids and asparagine. It has been assumed that the intermediate forerunner of asparagine in the plant is aspartic acid, and that an enzyme, for which the name asparaginase has been used, controls the reaction



The presence of aspartic acid in the plant had never been demonstrated until Kiesel [1924] claimed to have isolated it in the course of a separation of the nitrogen compounds of ripening rye ears. As no asparagine was found even in the later stages of ripening, Kiesel considered that aspartic acid cannot be the forerunner of asparagine. It is probable, however, that the reverse reaction is here occurring—that there is a breakdown of asparagine to aspartic acid; but instead of the deamidation only taking place at a stage still earlier than those investigated, as Kiesel suggests, it is more likely that there is a continuous diffusion of asparagine into the grain with almost simultaneous deamidation. Schulze and Winterstein [1910] came to the conclusion that asparagine migrates into the ripening seed where it is rapidly utilised for the production of protein.

In the earlier experiments on the autolytic splitting of ammonia from plant material, the whole process was referred to as one of "desamidation," and the inability to distinguish between the processes of deamination and deamidation, *i.e.* the removal of ammonia from the amino- and amide-groupings respectively, has given rise to considerable confusion and controversy. The enzymes responsible for the reactions leading to the liberation of ammonia were indiscriminately termed amidases or de-amidases, and these expressions are still often employed.

Many attempts have been made to effect a definite enzymic deamidation of asparagine by means of plant material. The only instance, however, in which this is claimed to have been achieved is that recorded by Kiesel [1909]. By the autolysis of etiolated seedlings or of the expressed juice, the whole of the asparagine originally present was broken down. The time period of four months, however, was somewhat excessive, for asparagine solutions, especially in the presence of plant material, are extremely susceptible to bacterial infection. It was not stated whether any sterility tests were carried out at the end of autolysis. The products formed by the breakdown of the asparagine were not identified and the attempt to obtain a preparation active on asparagine by precipitation of the expressed juice with alcohol was unsuccessful.

Schulze and Winterstein [1910] tried to detect in unripe pea and bean seeds an enzyme which would liberate ammonia from asparagine, but without success; in both cases practically the same amount of asparagine was recovered in the active and control experiments. Kato [1911] found that the press-juice of bamboo shoots contained an enzyme which would liberate ammonia from asparagine. The amount formed, however, was almost inappreciable, being of the order of 0·3 mg. from 0·2 g. of asparagine after 50 hours' incubation.

The presence of "amidases" in moulds was first demonstrated by Shibata [1904] who found in the mycelium of *Aspergillus niger* an enzyme which acted on certain amides including asparagine. The ammonia set free in the case of asparagine was only about 0·5 %. Pringsheim [1908], using an acetone-dried preparation of yeast, was unable to obtain any "desamidation" of amino-

acids or asparagine and considered that the ability of moulds to liberate ammonia from nitrogenous substances was essentially associated with a living condition of the mould.

The "de-amidases" of yeast were briefly dealt with by Dernby [1917] in connection with a study of the yeast proteolytic enzymes. Yeast press-juice was found to be inactive on amino-acids but asparagine was readily broken down. As the ammonia formed was always less than 50 % of the total nitrogen, it was concluded that it had been set free by the yeast ereptase from the $—CO.NH_2$ group which was regarded as a peptide linkage. This point will be discussed later in connection with some results obtained in the present research.

Effront [1917], in the course of a discussion on amidases, describes the preparation of a "butyro-amidase" which acted on acetamide and asparagine with liberation of ammonia. When a solution of asparagine only was used, the action was limited to the amide group; if, however, oxidisable substances were also present—*e.g.* products of proteolysis—then the amino-group of the asparagine was also attacked. This is one of the very few doubtful instances in which the amino-group has been considered to have been displaced by enzyme activity apart from the living cell. In the rather vague account of his experiments on butyro-amidase, Effront makes no reference, unfortunately, to the original publication of the results which are quoted.

In the sphere of animal physiology, the earliest investigations on the existence of amidases in the tissues of the animal organism were attended by the same lack of differentiation between deamination and deamidation; resulting from this, divergences of opinion and conflicting results are again observed. S. Lang [1904] found that minced organs were able to effect the removal of ammonia not only from asparagine but also from certain amino-acids. Abderhalden and Schittenhelm [1906], using the press-juice from liver, obtained no action on amino-acids. Fürth and Friedmann [1910], experimenting with various organs of the horse and pig, found that, in general, they were all capable of bringing about *in vitro* the hydrolysis of the amide group of asparagine. Further, the amounts of ammonia formed were usually no greater than could be accounted for by the amide nitrogen. It was suggested that the ability to break down asparagine was related to the general autolytic processes taking part in the physiological degradation of proteins.

More recently, Clementi [1922] carried out a systematic study of the enzymic deamidation of asparagine by the animal organism. Contrary to the findings of Fürth and Friedmann, Clementi asserts that the enzyme, for which he uses the name "asparaginase," is present only in certain animals and in certain tissues. It is interesting to note that whereas Fürth and Friedmann obtained the maximum activity with intestinal mucous membrane of the horse, Clementi, using the same material, found only a slight deamidation. The intestine of the pig was found to be quite inactive, but this result was not confirmed in the present research. With regard to the physiological significance

of the appearance of asparaginase in the animal organism, Clementi elaborated the theory that this enzyme is related, not to the processes of metabolic degradation of the protein molecule, but rather to the nature of the alimentation of the species, and that the phenomenon is one of biochemical adaptation of the organism to the presence or absence of asparagine in the food. The experimental data obtained by Clementi appear to conform very well to this attractive hypothesis.

From the above introductory review it is apparent that the only clear-cut evidence for the occurrence of the enzymic deamidation of asparagine apart from the living cell is that obtained by experiments with animal material. Even these investigations, however, have had as their object merely the proof of the existence of an enzyme capable of liberating ammonia from asparagine. No attempt has been made to furnish experimental proof that the product of the deamidation of asparagine is aspartic acid and, except in the researches of Clementi, no active preparation of the enzyme by alcohol or acetone precipitation has been obtained. Further, the factors which condition the activity of the enzyme have received little attention. Clementi found that a slightly alkaline medium was favourable but did not determine the optimum p_H for the enzyme or the range over which it was active.

In the higher plant even the existence of this enzyme has not yet been satisfactorily demonstrated, although the deamidation of asparagine is a phenomenon of the greatest importance in the metabolic processes of the plant. The primary object of this research, therefore, was to prove conclusively the presence of this enzyme in the higher plant; a further consideration was the need of obtaining some information as to the nature of the enzyme.

Experimental.

General methods.

In the following experiments, the young rootlets (culms) of germinated barley were used as the material from which to obtain the enzyme and investigate its nature. The barley was obtained from the malthouse (Messrs Watney, Combe and Reid, Mortlake) in the fresh or "green" condition, *i.e.* before the kilning process. It had been allowed to germinate 8–9 days and the rootlets were 1–1·5 cm. long. For the experiments on asparagine, a $N/10$ solution containing 15 g. per litre was used throughout; the solution contained 0·5 % cresylic acid (tricresol) as antiseptic except where otherwise stated. Cresylic acid was used in preference to toluene partly because the trouble of separating the toluene before estimation was avoided and, more especially, because it had been found by other investigators to be more efficient. In general, the experiments were carried out at 37° and the ammonia formed was estimated by distillation with magnesia and absorption of the ammonia in $N/10$ HCl.

The amount of asparagine broken down in the course of the distillation is very small; for 10 cc. $N/10$ asparagine the blank titration is approximately only 0·3 cc. This was always accounted for in the parallel control experiments which were carried out under the same conditions except that the enzyme had been destroyed by previously heating the material.

Activity of barley culms on asparagine.

For a preliminary test of activity, 4 g. of fresh roots were picked off from 100 g. of the barley and ground to a thin cream with water to 25 cc. The activity of the fresh roots is shown in Table I, 5 cc. of the preparation being equivalent to 0·8 g. of roots. As the separation of the fresh culms by hand picking was an extremely tedious and lengthy process, the remainder of the germinated barley was spread out in an incubator at 37° and allowed to dry for 3 days. The dried culms were then fairly easily separated by vigorously shaking the barley and afterwards sifting through a coarse mesh. From 9·5 kg. of the dried barley, 200 g. of culms were obtained and ground to a fine powder. By comparison with the yield of fresh culms, it is seen that the culms had lost at least half their weight by drying. If the enzyme had been unaffected by the drying process, therefore, the dried culms should have had about double the activity of the same weight of fresh culms. Actually the activity of the dried culm powder was certainly no greater than that of the same weight of fresh material. 10 g. of the root powder were ground to a thick paste with water to 80 cc. and 5 cc. (= 0·6 g. of roots) used for the activity tests given in Table I.

Table I. *Activity of fresh and dried barley roots on asparagine.*

(Maximum ammonia formation = 10 cc. $N/10$.)

	Time (hours)	cc. $N/10$ NH_3
Fresh roots:		
5 cc. preparation + 10 cc. $N/10$ asparagine + 5 cc. buffer p_H 7·5	88	8·75
5 cc. preparation + 10 cc. 0·5 % tricresol + 5 cc. buffer p_H 7·5	88	0·7
5 cc. (boiled) preparation + 10 cc. $N/10$ asparagine + 5 cc. buffer p_H 7·5	88	0·6
Dried roots:		
5 cc. preparation + 10 cc. $N/10$ asparagine + 5 cc. buffer p_H 7·5	88	7·5
5 cc. preparation + 10 cc. $N/10$ asparagine + 5 cc. buffer p_H 7·5	161	8·55
5 cc. preparation + 10 cc. 0·5 % tricresol + 5 cc. buffer p_H 7·5	161	1·45
5 cc. (boiled) preparation + 10 cc. $N/10$ asparagine + 5 cc. buffer p_H 7·5	161	1·25

Enzyme preparation by alcohol precipitation. 200 g. of dried barley culms were ground with 1½–2 litres of water for about 2 hours, sand being added to facilitate the grinding. The mass was wrapped in a thick filter-cloth and pressed out in a Buchner press. The residue was returned to the mill, ground with a further 1½ litres of water for 1 hour and again pressed out. The combined extracts measuring 2½ litres were centrifuged, and the clear liquid was precipitated with 4½ litres of ice-cold 90 % alcohol. The precipitate was centrifuged off

and washed with absolute alcohol and twice with ether, being centrifuged after each washing. The precipitate was air-dried as far as possible by breaking up in a small mortar, and the last traces of ether removed in a vacuum desiccator. Those steps in which the enzyme was in the presence of alcohol were carried out as rapidly as possible in order to minimise inactivation. The pale creamy-brown powder thus obtained weighed 8·85 g. or 4·4 % of the original dried root material; it was only partially soluble and when ground with water gave a thin suspension which slowly deposited. This alcohol precipitate is referred to below as the enzyme preparation.

To determine the activity of the preparation, 0·5 g. were ground with water to 25 cc. and 5 cc. of this suspension (= 0·1 g. of preparation) used for each of the experiments represented in Table II. Since 0·5 g. of the dried roots used for this preparation produced a break-down of 5·8 cc. $N/10$ (corrected) in 144 hours under the same conditions, it can be roughly calculated that the activity of the enzyme preparation was about 4–5 times that of the whole roots.

Table II. *Activity of the enzyme preparation.*

(Maximum ammonia formation = 10 cc. $N/10$.)

No.	Time (hours)	cc. $N/10$ NH_3
1. 5 cc. suspension. 10 cc. asparagine. 5 cc. buffer p_H 7·5	50	3·0
2. 5 cc. suspension. 10 cc. asparagine. 5 cc. buffer p_H 7·5	118	5·3
3. 5 cc. suspension. 10 cc. asparagine. 5 cc. buffer p_H 7·5	170	6·7
4. 5 cc. suspension. 10 cc. asparagine. 5 cc. buffer p_H 7·5	218	7·8
5. 5 cc. (boiled) suspension. 10 cc. asparagine. 5 cc. buffer p_H 7·5	218	0·8

The activity of the enzyme preparation under sterile conditions was confirmed, both in the presence of toluene and of cresylic acid. The extent of hydrolysis was practically the same as that obtained under the ordinary experimental conditions. The sterility tests were kindly carried out by Dr Wright and Mrs Channon of the Bacteriological Department, University College Hospital Medical School.

Isolation of aspartic acid as the end-product of the deamidation of asparagine. 2 g. of the enzyme preparation ground with water were added to 150 cc. $M/5$ asparagine solution (containing 0·5 % tricresol) together with 1·5 cc. of 2 N Na_2CO_3. The volume was made up to 200 cc. in a stoppered measuring cylinder and incubated at 37°. 5 cc. portions were drawn off and estimated at intervals until the maximum theoretical amount of ammonia was nearly obtained.

The results of these determinations are given in Table III; the action is slower than usual and this may be due to the higher concentration of tricresol in the mixture. The values for the velocity constant (monomolecular), calculated after allowing for an arbitrary blank of 0·6 cc., are also given. The slight fall in the values has been noted in the case of other enzymes, *e.g.* trypsin and lactase, and is due in part to the effect of the products of the reaction.

Table III.

Time (hours)	cc. $N/10$ NH_3 (max. = 7·5 cc.)	k
46	1·6	229×10^{-5}
90	2·3	207
164	3·3	192
260	4·5	189
380	5·7	188
520	6·65	179
640	7·35	176

At the end of the 27 days, the enzyme material was precipitated from the remainder of the mixture by addition of alcohol. The solution was evaporated to about 50 cc. and the aspartic acid precipitated with the requisite amount of HCl. After standing in the ice-chest overnight, the aspartic acid was filtered off, washed with 50 % alcohol, absolute alcohol and ether successively, and finally dried in a desiccator. 1·96 g. were thus obtained. By concentrating the mother-liquor and adding an equal volume of alcohol, a further 0·46 g. was obtained. The total yield was therefore 2·42 g. or 76 % of the theoretical 3·2 g. The loss is due in part to an incomplete separation of the aspartic acid and possibly, also, to anhydride formation. The crude aspartic acid on recrystallisation gave 2·16 g.

Analysis.

1. *Amino-nitrogen* (Van Slyke). Found 10·38 %, calculated 10·53 %.
2. *Total nitrogen* (Kjeldahl). Found 10·57 %, calculated 10·53 %.
3. *Formaldehyde titration* (method of L. G. Harris). The solution of aspartic acid used contained 0·4018 g. per 100 cc.

	cc. $N/10$ NaOH titrating to phenolphthalein
1. COOH *estimated*	
(*a*) 5 cc. of aspartic acid solution + 50 cc. alcohol + 10 cc. neutralised formaldehyde	2·92 (corr.)
	Theory 3·02
	cc. $N/10$ HCl titrating to methyl red
2. NH_2 *estimated*	
(*b*) Back titration of solution (*a*)	1·4 (corr.)
	Theory 1·51

Relation between quantity of enzyme and extent of hydrolysis. Fig. 1 shows the curve obtained by plotting the corrected values of the percentage hydrolysis against enzyme concentration. The extent of hydrolysis is fairly directly proportional to the quantity of enzyme preparation, but the curve falls off slightly at the higher concentrations.

Effect of temperature on activity. For this experiment a new enzyme preparation was used, 0·1 g. being taken for each estimation; both active and control experiments were carried out in duplicate at each temperature.

The curve obtained by plotting the corrected mean values of the percentage hydrolysis is shown in Fig. 2. The preparation used was unfortunately of a low activity but the curve shows that the optimum temperature for the enzyme is about 38°. The time period for this curve was 120 hours.

Effect of p_H on activity. As this series was started at the same time as the previous one, the same preparation was used, but the time of incubation was increased to 12 days. 0·1 g. of enzyme ground with water to a thin suspension

was added to 10 cc. of the asparagine solution and 5 cc. of the buffer indicated in each case. The actual p_H of each mixture was determined electrometrically after equilibrium had been reached in the course of some hours. The preparation had a very high buffering power, this being the more in evidence the further the p_H from neutrality. The optimum p_H was found to be approximately 7; the phosphate buffer was more favourable for the enzyme than the phthalate or borate buffers at the same p_H. Fig. 2 shows the curve obtained by plotting the actual p_H values against the corrected mean figures for percentage hydrolysis.

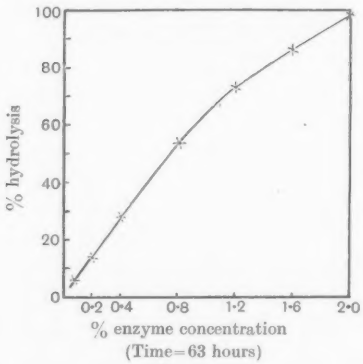


Fig. 1.

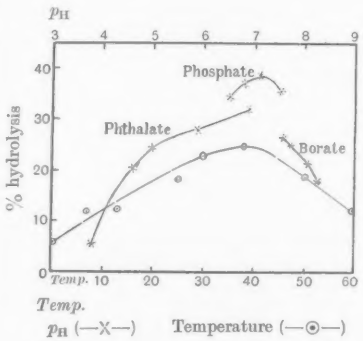


Fig. 2.

Specific action of the enzyme preparation on l-*asparagine.* The asparagine used in the previous experiments was the naturally occurring laevo-form with which the enzyme normally has to deal. It was interesting to find out whether the enzyme was specific for this form or was also capable of effecting the hydrolysis of the dextro-modification. It has been shown by Bergell and Wülfing [1910] that the laevo-component of *dl*-leucinamide is hydrolysed by pancreatin very much more readily than the dextro-form.

A preparation of asparagine containing an excess of the dextro-component was prepared according to the method of Pringsheim [1910]. The rotation in 10 % HCl was $[\alpha]^{18°}_{\text{Hg green}} = -11{\cdot}31°$ as compared with $+38{\cdot}8°$ for the natural laevo-form. Table IV shows the hydrolysis of a solution containing 0·6202 g. per 50 cc. by a water extract of the whole barley roots. Only about 50 % of the maximum ammonia formation was obtained, and it would appear

Table IV. *Activity of enzyme preparation on a mixture of* d- *and* dl-*asparagine.*

(Maximum ammonia formation = 4·2 cc. *N*/10.)

Time (hours)	cc. *N*/10 NH_3. Active	Control	Corrected
44	4·4	2·6	1·8
68	4·6	2·6	2·0
92	4·7	2·6	2·1
212	4·8	2·6	2·2

that the enzyme has little or no activity on the dextro-form of asparagine. Ravenna and Bosinelli [1919] showed that when moulds are grown on a mixture of *d*- and *l*-asparagine, the laevo-form is readily attacked but the dextro-form is unaffected.

The specificity of the enzyme preparation and the nature of deamidation. The simple amides, acetamide and propionamide, were entirely resistant to the action of the enzyme, but *d*-glutamine, which is closely associated with asparagine both in structural form and in its natural occurrence, was hydrolysed fairly rapidly. In these experiments the ammonia estimations were carried out by aeration with potassium carbonate in the cold. It is interesting to note that urea also was broken down but at a very much slower rate than with soya-bean urease.

With the object of obtaining some information on the nature of the enzyme and of the process of deamidation, the experiments described below were carried out. In order to ascertain whether deamidation is related to the processes underlying protein degradation, the action of the proteolytic enzymes pepsin, trypsin and, more particularly, erepsin was investigated.

Pepsin and trypsin were both found to be inactive on asparagine; the inactivity of trypsin had been previously demonstrated by Schwarzschild [1904].

A preparation of pig erepsin, however, proved to be readily active on asparagine, although Clementi's results with this material were negative. Bergell and Wülfing [1912] in their experiments on the splitting of leucineamide by pancreatin concluded that there was present a specific enzyme responsible for the amide cleavage. Levene, Simms and Pfaltz [1926], using intestinal erepsin, were able to obtain a slight hydrolysis of glycineamide. In a recent study of the peptidases, Waldschmidt-Leitz and his collaborators [1927] found that pig erepsin had a slight activity on glycineamide but hydrolysed leucineamide fairly readily.

The results obtained by acting on asparagine with an aqueous-glycerol extract of the mucous membrane of pig intestine are given in Table V. The p_H values in each case were measured electrometrically. It is seen that below p_H 6 the enzyme has only a very slight activity and this is the minimum p_H for the action of erepsin on dipeptides. The control figures were obtained by using the same amount of boiled extract.

Table V. *Action of pig erepsin on asparagine.*

(Maximum ammonia formation = 10 cc. $N/10$.)

p_H	Time (hours)	cc. $N/10$ NH_3		
		Active	Control	Corrected
3·0	68	2·15	2·2	0
4·0	68	2·35	2·2	0·15
5·1	68	4·0	2·2	1·8
6·0	68	7·4	2·2	5·2
7·1	50	7·4	2·25	5·15
8·0	50	8·7	2·25	6·45

From a consideration of these results, the question arises whether the enzyme in the barley preparation responsible for the splitting of the amide group of asparagine is merely a plant erepsin or—to use the preferable nomenclature—a peptidase. The action of the barley enzyme on a dipeptide (glycylglycine) was therefore investigated.

Activity of the barley enzyme towards glycylglycine.

For each estimation 10 cc. of barley root extract (containing 0·2 % tricresol) were added to 2 cc. of *M*/10 glycylglycine together with 0·25 cc. of *N*/10 NaOH to raise the p_H to 7·6. At the end of the indicated time at 37°, 40 cc. of alcohol were added and the mixture filtered. The filtrate was titrated with *N*/10 NaOH forward to phenolphthalein and with *N*/10 HCl back to methyl red. Table VI shows the increase in the titration values over the initial values showing that the hydrolysis has proceeded up to 70 % of the theoretical maximum.

Table VI. *Action of barley enzyme on glycylglycine.*

(Maximum value for both titrations = 2 cc. *N*/10.)

Time (hours)	Titrations, cc. *N*/10	
	Forward	Back
0	0·0	0·0
20	0·6	0·55
24	0·75	0·65
48	0·95	1·85
72	1·30	1·20
96	1·50	1·30

Discussion.

The results of the foregoing experiments prove beyond question the existence in the higher plant of an enzyme capable of effecting the deamidation of asparagine. The fact that its action had not previously been satisfactorily demonstrated is probably due to the comparatively low activity of the enzyme or to the difficulty of obtaining it in a sufficiently concentrated form.

With regard to its identity, the experiments on the hydrolysis of glycylglycine and on the behaviour of the proteolytic enzymes towards asparagine would seem to indicate that we are here dealing with an enzyme in the nature of a plant erepsin. This is in accordance with the suggestion made by Dernby from his results on the hydrolysis of asparagine by autolysed yeast. As was pointed out in the introduction, Dernby considered that the amide group of asparagine was to be regarded as similar to a peptide linkage and that it was the yeast ereptase which was responsible for its cleavage.

The enzyme which breaks down asparagine has hitherto been included in the group of amidases under the name of asparaginase. The nomenclature of this group is discussed by Oppenheimer [1926] who separates the amidases into two sections. Those which are responsible for the liberation of ammonia from amino-acids—and which have a very doubtful existence apart from the living cell—he terms *amino-acidases*.

The name *amidases* he retains for the enzymes which, in general, are capable of hydrolysing the —CO.NH_2 grouping only. There is, however, still no sharp line of demarcation between this group and the peptidases to which the cleavage of the peptide linkage —CO.NH— is due.

Levene and Waldschmidt-Leitz have both probably assumed the peptide nature of the amide group in studying the action of peptidases on such amides as glycineamide as well as on true dipeptides. By adopting the view that the amide group of asparagine is essentially a peptide linkage and that both asparagine and glycylglycine are hydrolysed by a barley peptidase, our conception of the protein metabolism of the plant is considerably simplified. Asparagine need then no longer be regarded as a substance apart and distinct from the usual products of protein metabolism but simply as a dipeptide.

If the deamidation of asparagine is indeed due to a peptidase, as is here considered to be the case, then the conclusions of Clementi on the specific distribution of "asparaginase" in the animal kingdom would have very little significance. Since erepsin is an essential factor in the protein metabolism of all animals, the ability to break down asparagine should be universal.

Summary.

A review of the literature shows that in spite of the great importance of asparagine in plant metabolism the enzyme responsible for its deamidation had not been satisfactorily demonstrated.

This enzyme has been shown to be present in the rootlets of germinating barley, and an active preparation has been obtained by alcoholic precipitation of the aqueous extract. It appears to be specific for the naturally occurring *l*-asparagine. The optimum p_H is about 7 and the optimum temperature about 37°.

A preparation of pig intestinal erepsin was found to be active on asparagine. As the barley preparation is able to effect the hydrolysis of glycylglycine a peptidase (barley erepsin) must be present.

It is concluded that the asparagine is also hydrolysed by this enzyme, and that it is unnecessary to assume the presence of any specific amidase (asparaginase).

We wish to thank the Government Grant Committee of the Royal Society for a grant in aid of expenses.

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CXVII. NOTE ON THE UNSAPONIFIABLE MATTER OF YEAST-FAT.

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(*Received July 1st, 1927.*)

During an investigation on the phospholipins of yeast, the results of which were recently described by us [1927], we determined the iodine value of the total unsaponifiable matter of yeast fat. The particular specimen examined gave an iodine value of 95·5: about 50 % of this material consisted of a sterol precipitable by digitonin and giving iodine values lying between 188 and 200. We therefore made the deduction that the unsaponifiable matter consisted partly of sterol and partly of a saturated substance.

Further investigation of the unsaponifiable matter shows that this deduction is not generally true for most of the specimens of unsaponifiable material which have been subsequently examined. One reason for this is that the iodine values of the unsaponifiable matter vary widely according to the particular sample of yeast employed and according to the method used for the isolation of the unsaponifiable matter. After removal of the sterol precipitable by digitonin, an oil may be obtained giving a very high iodine value.

It was shown by Smedley-MacLean and Thomas [1920] that the iodine values of the sterols determined by Wijs' reagent gave variable values often twice as great as those determined by means of Hubl's reagent. Even with the latter reagent abnormally high values may be obtained, and our recent results have confirmed the opinion that, in this case too, variable results may be obtained unless the conditions are very carefully controlled.

That part of the unsaponifiable matter of yeast-fat which is not precipitated by digitonin appears to vary considerably in its composition, and its degree of unsaturation is very largely influenced by the particular method of saponification used and by the subsequent treatment the material undergoes. Other highly unsaturated and probably labile substances are present in varying proportion. An investigation is at present in progress which it is hoped will throw some light on the nature of the complex mixture of substances included under the designation of the unsaponifiable matter of yeast-fat.

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CXVIII. VITAMIN A FORMATION IN THE ETIOLATED WHEAT SHOOT.

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As the result of extensive feeding experiments with green plant tissues, etiolated tissues, and seeds, Coward and Drummond [1921] established the superiority of green tissues as a source of vitamin A (not then differentiated from vitamin D). Very small amounts of vitamin were found in seeds and in etiolated tissues, which led to the inference that vitamin A formation in the growing plant was to be associated with the presence of chlorophyll. The vitamin was to be considered a product of photosynthesis.

A year later Wilson [1922] contradicted this view. Experiments were described in which the dried powdered stems of wheat grown to a height of 3 in. in the dark, and fed to rats at rates of 5 and 8 % of the basal ration in preventive and curative experiments respectively, were found to be as effective as the same amounts of dried powdered green stems in permitting growth on a diet deficient in vitamin A. Wilson concluded that light was unnecessary for the formation of the vitamin, and that if green shoots contained more vitamin A than similar etiolated shoots this was in all probability due to the acceleration of the physiological processes of the plant during photosynthesis.

To these conclusions Coward [1923] advanced the objection that no control experiments had been carried out to test the vitamin content of the seeds from which Wilson's etiolated shoots had been derived, a criticism which gained weight through the presence in his basal diet of 20 % of lard, which might have contained traces of vitamin just insufficient to support growth, but sufficient when supplemented by traces of vitamin from the seeds. Coward still maintained that light was necessary for the formation of the vitamin, but as the result of further experiments admitted that the presence of chlorophyll was not essential. Vitamin A formation could proceed in the absence of carbon dioxide or oxygen, but was prevented by the presence of chloroform.

By some authorities [Accessory Food Factors Committee, 1924; McCollum and Simmonds, 1925] the results of Coward and of Wilson have been considered contradictory, but while this is true of the conclusions reached the actual experiments seem to have been quite incomparable. Wilson's 5 and 8 % allowances of powdered stems may be calculated to have been derived from wheat equivalent to 18 and 29 % of the diet, and on a food intake of 10 g. 125 and 200 stems must have been fed daily to each rat. In reply Coward

quoted experiments in which the daily dose was limited to three etiolated shoots, or to the unsaponifiable matter from fifteen. Even at such a low level slight growth was observed in some cases.

Further experiments by Coward [1925] led to no change in her position, which was approved by Rosenheim and Drummond [1925]. Hess and Weinstock [1924], however, had shown that fresh etiolated wheat shoots, even when fed to rats at the rate of 10 g. daily, were ineffective in curing rickets, and it was obviously desirable to repeat the experiments with vitamin D, so intimately connected with sunlight, supplied by the irradiation of the diet, or some other means. By using a technique so modified and by increasing the daily allowance of shoots from three to five, Coward [1927] has recently detected the presence of appreciable quantities of vitamin A in etiolated wheat shoots, and has accordingly abandoned the view that light is necessary for the formation of the vitamin.

The experiments to be described, which were nearing completion when Coward's results were published, afford independent confirmation on this important point.

Experimental.

The wheat seeds used weighed on an average 20 to the g., and seem therefore to have been of a larger variety than those used by Wilson, which weighed 68 to the g. They were sprouted in moist sand contained in zinc trays, which were arranged in a wooden box. The box was painted black externally and the open side, by which the trays were inserted, was covered by a flap of four thicknesses of black calico, held in position by elastic bands. In the routine finally adopted all sowing, collection, and feeding was carried out after nightfall. Each tray accommodated seeds to provide shoots for 2 days' feeding. Sowings were accordingly performed on alternate days, the sand being strongly heated before each sowing to destroy bacteria. Sprouting was continued for 10 days, the shoots obtained varying somewhat in size in the different batches, but as a rule having stems about 4 in. long. In colour they were pale yellow.

Young rats, of weights from 42 to 60 g., were "run out" on the customary basal diet of this laboratory, 0·25 cc. of marmite extract and 0·2 cc. of lemon juice per rat per day supplying vitamins B and C respectively. Vitamin D was supplied by the administration of daily doses of 5 mg. of irradiated cholesterol from the points indicated on the accompanying growth curves. Out of a dozen rats the four weakest were used as positive controls (not shown), the remaining eight were given either etiolated shoots or seeds at the points indicated on the curves (Fig. 1), one bar corresponding to ten shoots or seeds, two bars to twenty, and three to thirty.

The four rats receiving seeds declined rapidly in weight, became rough-coated and emaciated, developed respiratory troubles and either died or were killed in dying condition. The rats receiving etiolated shoots, at first fed in a dark room in advance of the basal ration, remained comparatively smooth-

coated. Three rats (nos. 2, 3 and 4) fluctuated in weight, though gaining on the whole. The fourth (no. 1) showed a serious decline and developed respiratory troubles, possibly through delay in feeding the shoots. Unfortunately some of the animals did not regularly ingest all the shoots offered, usually nibbling the grain and discarding the stem and roots: it therefore became necessary to modify the routine. Thirty entire shoots were now allowed to each animal, the stems, roots, and grains being finely chopped and mixed intimately with the basal ration, which was fed after nightfall and consumed before morning. In addition, the rats were irradiated before a mercury arc lamp for 10 minutes daily.

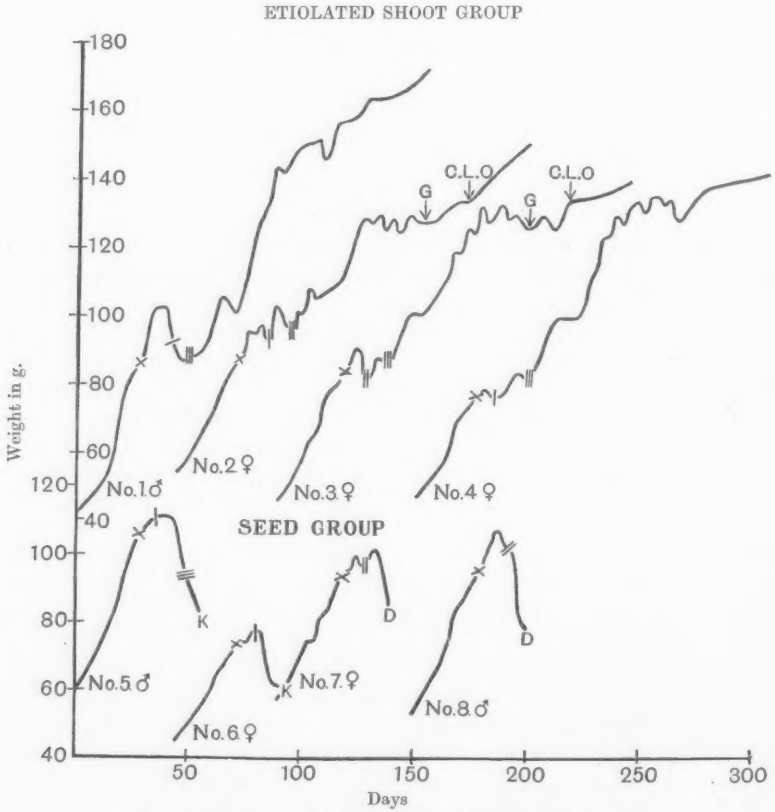

Fig. 1. Formation of vitamin A in the etiolated wheat shoot.

×=Feeding of irradiated cholesterol commenced.

|, ||, |||=10, 20 or 30 seeds or etiolated shoots fed *per diem*.

K=killed. D=died. G=30 green shoots fed *per diem*.

C.L.O.=2 drops of cod-liver oil replaced shoots.

As the result of such treatment growth was restored in all four cases, demonstrating beyond doubt the activity of the etiolated wheat shoots in repairing deficiency of vitamin A. At the time of the adoption of the revised routine, however, only two animals of the control group remained alive. Two more rats were therefore placed on the basal diet, receiving in addition an allowance of thirty wheat seeds each per day and direct irradiation from the commencement of the experiment. After the normal period they declined and died, one displaying severe symptoms of xerophthalmia.

Discussion.

Since feeding was conducted after nightfall the shoots were exposed only to artificial illumination before ingestion by the rats. It seems improbable that illumination of such feeble intensity should cause a formation of the vitamin. The rats themselves, however, were not kept in the dark, and a loophole for photosynthetic action remains open if the formation of the vitamin, as in the case of vitamin D, be pictured to take place in the animal on exposure to diffuse daylight or to mercury arc illumination. Further experiments are contemplated to clear up this point. For the present there is no reason to believe that vitamin A is not produced by the plant in the dark. If it is not formed under such conditions a precursor must at least make its appearance, since wheat seeds fed under similar conditions of illumination were found to be ineffective.

From the growth curves it will be seen that for a period of several weeks all the rats receiving etiolated shoots grew at an approximately normal rate. In the case of rat no. 1 (male) such growth was continued without serious check for about four months, a weight of 172 g. being attained 115 days after the addition of shoots to the diet. The remaining rats (nos. 2, 3 and 4 (females)) after a time ceased to grow, marked plateau periods in the growth curves being attained at body weights of about 130 g. The cause of this stoppage is not known. Although no acute symptoms, beyond occasional sneezing, were observed, all the four rats were obviously in an imperfect state of nutrition, rib bones being unduly prominent, coats slightly rough, backs slightly arched and muscular tone poor. A partial insufficiency of the vitamin might well be suspected, but shoots were fed at six times the minimum determined by Coward; moreover the replacement of etiolated shoots successively by green shoots and by cod-liver oil in the diets of two of the rats, while promoting slight growth, did not notably improve their condition above that of the others. Whatever the cause of such imperfect nutrition may have been, however, it would appear certain that the etiolated shoots, directly or indirectly, provided an active source of vitamin A, since the rats could certainly not have survived the control animals for several months without any of the characteristic signs of deficiency in the continued absence of that vitamin.

In the present experiments, it should be observed, shoots at an advanced stage of development were employed. In experiments with wheat and corn

seeds germinated for shorter periods (20–48 hours), Coward [1927], Harrow and Krasnow [1922, 1924], and Karshan, Harrow and Krasnow [1927] have failed to establish any increase in vitamin A content above that of ungerminated seeds, and it has been suggested that the vitamin is not synthesised under such conditions. In Coward's experiments, however, only five germinated wheat seeds were allowed per rat per day, a level dangerously low in view of past experiences at a three-shoot dosage, while in those of the American workers vitamin D was not supplied. Such evidence, therefore, while of obvious importance from an economic standpoint, cannot afford an adequate basis for the absolute denial of the formation of traces of vitamin A, and for the present the question should be left open. Stepp [1925], it may be mentioned, has reported the synthesis of traces of vitamin A in wheat seeds germinated for 4 days, but as no precautions were taken to exclude the action of light this finding cannot be considered relevant to the problem under discussion.

SUMMARY.

Fresh etiolated wheat shoots, 10 days old, fed to rats receiving a diet deficient in vitamin A, but adequate in vitamin D, at the rate of thirty shoots per day (equivalent to 1·5 g. of dry wheat) prevented death and restored growth. Seeds fed at the same rate were ineffective.

This result is in agreement with statements by Wilson [1922] and Coward [1927] that vitamin A may be formed in etiolated tissues. In the present experiments the feeding of shoots was continued for some months after the death of the control animals. The final condition of the animals was fair, although they appeared somewhat lean and rough-coated when compared with similar rats receiving a liberal diet.

My thanks are due to Sir F. G. Hopkins for support and criticism.

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CXIX. THE INFLUENCE OF TEMPERATURE ON THE NATURE OF THE FAT FORMED BY LIVING ORGANISMS.

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(*Received July 5th, 1927.*)

Leathes and Raper [1925, p. 119] have put forward the view that the temperature at which fats are formed in nature is one of the factors which determine their degree of unsaturation. This suggestion is based mainly on three lines of reasoning. Firstly, a general survey of the fats found in animals and plants indicates that the highly unsaturated fats are most commonly found in plants growing in temperate or cold regions and in the poikilothermic animals which live under relatively cold conditions, whilst the most saturated fats are found in tropical or subtropical plants and in warm-blooded animals. The carnivora should not be included in this generalisation because the composition of their adipose tissue fat is partly, at any rate, determined by the fat which they take as food, and even in animals such as the pig, which produce fat easily from carbohydrate, differences have been observed in the composition of their stored fat which may be referred to differences in the composition of the fats present in the cereals on which they have been fed. Secondly, it is usual to find that the fat present in living organisms is fluid at the temperature at which the organisms exist. This would preclude the formation of saturated acids to any great extent in organisms living under temperate or cold conditions because such acids would tend to raise the melting points even of mixed glycerides which contained them. The higher melting point of the saturated acids would be less important from this point of view in the warm-blooded animals or tropical plants and it might be more likely therefore to expect saturated acids to occur to a greater extent in organisms living under warmer conditions. Thirdly, various hypotheses have been made regarding the chemical reactions by which fatty acids are formed in nature, *viz.* autocondensation of acetaldehyde [Leathes, 1906], condensation of pyruvic acid and acetaldehyde [Smedley and Lubrzynska, 1913], or autocondensation of pyruvic acid [Neuberg and Arinstein, 1921]. All these admit of the possibility of the formation of long chains of carbon atoms with unsaturated linkages as products of the condensation reactions which produce them and if they are to give rise to saturated carbon chains a reduction must take place subsequently.

If this represents what takes place, then the unsaturated acids would be the precursors of the saturated. Two different types of reaction are involved, namely, condensation and reduction. It seems possible that both these reactions would not necessarily take place with the same ease at low temperatures and, if it be assumed that the condensation takes place readily at low temperatures whereas the reduction requires a higher temperature, an explanation of the distribution in nature of the saturated and unsaturated acids referred to above might be found on these lines. This would not imply, as might at first sight be thought, that at any given temperature the exact degree of unsaturation of a fat would be determined solely by that temperature. It would also depend on other factors, *e.g.* the amount of the catalyst present which brings about the reduction, or the extent to which the synthesis into glycerides was taking place, thus possibly inhibiting further reduction.

It seemed to us that it would be possible to test the main hypothesis as to the influence of temperature, experimentally, by growing an organism at different temperatures, all other conditions being kept the same. Certain of the fungi seemed likely to be suitable subjects since they grow fairly readily at different temperatures and some of them contain notable amounts of fat. The work was actually begun three years ago but, owing to one of us (L. K. P.) having to withdraw owing to unforeseen circumstances, it had to be discontinued in 1926 and a further opportunity to continue it has not yet arisen. In the meantime, Terroine, Bonnet, Kopp and Véchot [1927] working on almost the same lines have obtained results in complete accord with our own. We have therefore thought it advisable to publish the data we have obtained up to the present. The work will be extended when a suitable opportunity offers.

Experimental.

Two fungi were used, namely, *Aspergillus niger* and *Rhizopus nigricans*. The former was grown on Czapek's solution to which 1 % ammonium sulphate and 3 % glucose were added. The latter would not grow on this medium but grew well in Meyer's solution with the addition of 1 % asparagine and 3 % glucose. The fungi were grown in litre flasks containing 500 cc. of the medium which were sterilised in the usual way. It was found possible to grow *Aspergillus niger* at three temperatures, namely, 18°, 25° and 35°, whereas *Rhizopus nigricans* was grown only at 12° and 25°, it being found impossible to grow this organism at 35°. The rate of growth varied with the temperature and it was allowed to continue till a good mat of the mould covered the surface of the medium. In some instances the fungus was left for some time after this had occurred in order to see what effect this would have on the amount of fat present and on its iodine value.

The methods used for extracting the fat from the mould were as follows. The mat of fungus was broken up, strained and pressed on a Büchner funnel and well washed with distilled water; after roughly drying between filter-

paper it was weighed. It was next covered with alcohol in a wide-necked flask and allowed to stand at least 24 hours. The alcohol was then removed by filtration and the fungus extracted with hot alcohol by suspending it in a thimble from the lower end of a reflux condenser which was attached to a flask containing 75 cc. boiling absolute alcohol. After extracting for 3 hours, the alcohol was removed from the flask and a further extraction with a fresh portion of 75 cc. alcohol was carried out. The three alcoholic extracts were combined, 15 cc. of 50 % aqueous KOH were added and most of the alcohol was distilled off on the water-bath, the fat undergoing saponification during this process. The fatty acids and unsaponifiable matter soluble in light petroleum were obtained from this solution by acidification and shaking with a measured quantity of light petroleum as in the usual method of fat estimation by the modified Liebermann method [cf. Leathes and Raper, 1925, p. 58]. From an aliquot part of the light petroleum extract, the fatty acids were removed by shaking with dilute sodium hydroxide in 50 % alcohol and then, after liberating the acids again with sulphuric acid, they were taken up in light petroleum, the solvent was distilled off in an atmosphere of CO_2, the flask finally evacuated for half an hour whilst in a boiling water-bath and the fatty acids weighed. The mean molecular weight was determined by titration in excess of alcohol with aqueous $N/10$ NaOH and, after recovery of the fatty acids from this solution by acidifying and extracting with light petroleum, their iodine value was determined by Wijs' method.

The results obtained are given in the accompanying table.

Fungus	Temp.	Time of growth (days)	Moist wt. of fungus (g.)	Total wt. of fatty acids (g.)	Mean molecular wt.	Iodine number	Mean iodine number
Aspergillus niger	18°	17	49·8	0·323	303	146·7	149
		17	46·3	0·206	300	153·6	
		56	39·9	0·084	323	150·2	
		56	41·5	0·086	330	145·9	
	25°	10	59·4	0·276	287	124	129
		14	50·3	0·302	304	132·5	
		17	51·1	0·254	290	131·3	
		35	40·5	0·252	287	127	
	35°	6	39·2	0·468	293	92·1	95
		7	38·8	0·527	285	92·3	
		9	37·8	0·633	290	99·9	
Rhizopus nigricans	12°	30	34·6	1·705	281	88·7	88
		30	31·0	1·669	289	87·2	
	25°	10	32·5	1·082	288	79	78
		13	35·4	1·168	287	77·3	

Discussion.

It will be seen, with both fungi, that the more unsaturated fats are produced at the lower temperatures, and, in the case of *Aspergillus niger*, the fat produced at a temperature intermediate between the highest and lowest had also an intermediate iodine value. The period of growth does not appear to affect the results at any given temperature. Comparable results have been

obtained by Terroine, Bonnet, Kopp and Véchot [1927] with the mould *Sterigmatocystis nigra*[1]. When grown at 35° the iodine value of its fatty acids was 85, whereas when grown at 17° it was 114. With the Timothy Grass bacillus the iodine value of the fatty acids was 33 when grown at 35° and 58 at 14°. These observers also determined the iodine value of the total fatty acids of a mollusc (*Melanopsis donneti*) and a fish (*Astatotilapia desfontainesi*) living at a temperature of 30°, the figures obtained being 91 and 87 respectively, whereas the fatty acids of a mollusc (*Limnaea peregra*) living at 8° had an iodine value of 125. These results all support the view that the temperature of the environment has an influence on the degree of unsaturation of the fatty acids which organisms produce. This however does not prove that all the deductions are correct which led to the hypothesis that temperature influences the nature of the fat formed in living organisms. Although in our own work, and that of Terroine and his colleagues, temperature was the only variable imposed by the experimenters on the different organisms, it was not necessarily the only variable in the experiments. It has been shown by the French workers that the composition of the organisms grown at different temperatures is not absolutely the same, though the differences are not very great. Then, difference in conditions of growth, *e.g.* temperature, the rate of growth, the type of medium, the exhaustion of the medium, affect the morphological character of fungi and have some influence on spore formation. These modifications of the life processes of the organisms imply that the chemical reactions which are normally taking place in them will be considerably modified. For these reasons it cannot be considered that the few experiments which have been carried out in this field of work prove that the changes observed in the composition of the fatty acids in organisms grown at different temperatures are simply the result of the effect of temperature on the two chemical reactions which have been thought to play a great part in fat formation, *i.e.* condensation of some simple aliphatic substance and reduction of the unsaturated compounds produced. Terroine and his colleagues, by comparing the energy loss from the medium during growth with the potential energy of the fungus formed at different temperatures, have shown that the expenditure of energy is somewhat greater at the higher temperatures. This indicates that the production of the more saturated acids under these conditions involves a greater utilisation of the potential energy of the medium and suggests that reduction succeeds condensation in the series of reactions by which fatty acids are produced, and thereby involves a greater expenditure of energy.

Summary.

1. The iodine value of the fatty acids produced by *Aspergillus niger* and *Rhizopus nigricans* varies with the temperature at which these organisms are grown.

[1] A synonym of *Aspergillus niger*.

2. At higher temperatures the fatty acids produced are more saturated, this being in conformity with the observation that, in general, the more saturated fats found in nature are usually produced at higher temperatures than the less saturated ones.

We desire to express our thanks to Professor Wilfred Robinson for pure cultures of the fungi used and to the Government Grant Committee of the Royal Society for a grant in aid of the expenses of this investigation.

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CXX. THE GLUCOSE METABOLISM OF KIDNEY TISSUE *IN VITRO*. I.

By JAMES TUTIN IRVING (*Beit Memorial Research Fellow*).

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(*Received July 7th, 1927.*)

Recent work on the relationship between glycolysis and respiration in normal tissues by Warburg and Meyerhof and their associates has shown that the changes previously found to take place in muscle, occur in most body cells. The experiments to be described below have been undertaken partly to determine more fully the part glucose takes in these changes, and also to see if the theory of surface activity previously adumbrated by me [1926] for blood corpuscles applies to other body cells. So far only the kidney has been investigated.

Experimental.

In all the experiments undertaken, fresh rabbit's kidneys were employed. They were perfused with warm saline till free from blood and the cortex was then cut off with small scissors and finely chopped with a razor. Weighed amounts of this were then placed in isotonic phosphate buffer at a p_H of about 8·0. The present writer has found that the best proportions of kidney tissue to phosphate buffer are one part of the former to four of the latter, and this proportion has been used throughout. Enough glucose (Kahlbaum) was added to bring the final concentration up to from 0·10 % to 0·20 %.

Sugar estimations were carried out on the supernatant fluid by the method of Hagedorn and Jensen. In the absence of glucose the reducing power did not change, or rose very slightly, while the distribution of glucose between buffer and kidney appeared to reach an equilibrium almost immediately. It thus appeared justifiable to attribute changes in reducing power to alterations in glucose content.

The tubes containing the suspension were completely immersed in a water-bath at 38° and were continually shaken by a mechanical device so that the contents were thoroughly mixed during the entire time of the experiment.

In several of the experiments, hydroxyquinoline sulphate was used in a concentration of 1 in 1500 as an antiseptic. Control experiments, in which the kidney tissue was removed from a suspension after incubation for 4 hours at 38°, and the phosphate-glucose solution reincubated, showed that the glucose content did not change subsequently. In addition, infection was not at all probable during the short period the experiments lasted, which seldom exceeded 4 hours, and in the majority of cases was no longer than two.

Factors governing the rate of glucose utilisation.

Immediately incubation was begun, the glucose content of the phosphate buffer was found to fall at a rate which was fairly constant for each specimen, but varied somewhat from kidney to kidney, the amount used % per hour being between 0·015 and 0·040 g. Exp. 1 in Table I and Fig. 1, curve 1, show results typical of many obtained. The value of k as calculated for a monomolecular reaction rises as the reaction proceeds, which would suggest that the rate was linear. The power of utilising glucose was found to be lost or greatly weakened if the kidney was kept for 24 hours at 0°.

In certain experiments, however, discrepant results were obtained which suggested that other factors might be playing a part. A series of experiments was undertaken in which the suspensions were incubated *in vacuo* in Thunberg tubes. The change in reducing power was estimated during this period, and the suspension was then placed in an aerobic tube and reincubated, and changes in the reducing power noted. Typical results of these experiments are shown in Table I, Exps. 2, 3 and 4, and Fig. 1, curve 2.

Table I.

Exp. no.	Conditions	Hours	Glucose %	Glucose utilised %	k
1	Aerobic	0	0·179	—	—
	,,	1	0·154	0·025	0·0645
	,,	2	0·129	0·050	0·0715
	,,	3	0·110	0·069	0·0707
	,,	4	0·092	0·087	0·0725
2	Anaerobic	0	0·199	—	—
	,,	2	0·202	0	—
	Aerobic	0	0·202	—	—
	,,	1	0·173	0·029	—
	,,	2	0·155	0·047	—
	,,	3	0·148	0·054	—
3	Anaerobic	0	0·226	—	—
	,,	2	0·217	0·009	—
	Aerobic	0	0·219	—	—
	,,	1	0·199	0·029	—
	,,	2	0·186	0·042	—
4	Anaerobic	0	0·195	—	—
	,,	1·5	0·195	0	—
	Aerobic	0	0·191	—	—
	,,	1	0·170	0·021	—
	,,	2	0·161	0·030	—

These figures show that the presence or absence of oxygen plays an important part in the utilisation of glucose, and that this can be completely stopped *in vacuo*, while, during the subsequent aerobiosis, glucose is again utilised. For these results to be consistently obtained, it is essential that the kidney be first thoroughly freed from blood, as otherwise enough oxygen is held by the haemoglobin remaining to promote some glucose utilisation.

The utilisation of glucose was also found to be markedly inhibited by cyanide. Experiments were undertaken using KCN in a final concentration

of $M/500$ and $M/250$. Before being used, the cyanide solution was adjusted to the same p_H as the phosphate buffer. Control experiments with KCN and kidney tissue in the absence of glucose showed that the reducing power did not change, and it was also shown that KCN did not interfere with the sugar estimations.

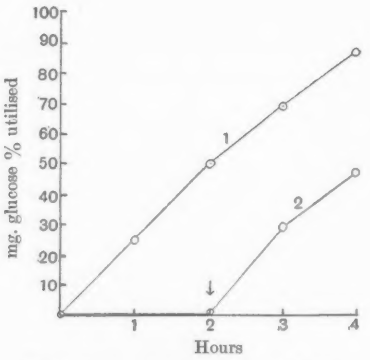

Fig. 1. The rate of utilisation of glucose.
Curve 1. Aerobic incubation.
Curve 2. Anaerobic and aerobic incubation. At the arrow, the suspension was transferred from an anaerobic to an aerobic tube.

Typical results are shown in Table II.

Table II.

		Control tube		Experimental tube		
Exp. no.	Hours	Glucose %	Glucose utilised %	Glucose %	Glucose utilised %	Concentration of KCN
1	0	0·157	—	0·163	—	$M/500$
	1	0·195	0·062	0·163	0	
	2	0·059	0·098	0·161	0·002	
	0	—	—	0·125	—	$M/250$
	1	—	—	0·129	0	
	2	—	—	0·131	0	
2	0	0·139	—	0·139	—	$M/250$
	1	0·106	0·033	0·132	0·007	
	2	0·081	0·058	0·125	0·014	
3	0	0·181	—	0·163	—	$M/500$
	1	0·145	0·036	0·159	0·004	
	2	0·119	0·062	0·173	0	
4	0	0·138	—	0·129	—	$M/250$
	1	0·104	0·034	0·129	0	
	2	0·079	0·059	0·129	0	
	0	—	—	0·139	—	$M/250$*
	2	—	—	0·129	0·010	

* 0·04 mg. Fe added (as used by Harrison [1924]).

In Exp. 4 iron was added as ferrous ammonium sulphate. This experiment, which is typical of several, shows that added iron does not influence the

inhibition by cyanide. It has also been found that KCN inhibits the glucose utilisation by unperfused kidneys to the same extent as by perfused, which would tend to show that iron is not acting catalytically.

The possibility cannot be disregarded that during these experiments in which no glucose appears to be utilised, an equal amount of other reducing bodies may be formed. Even if this were so, it would still show that glucose is differently utilised aerobically and anaerobically. The constancy of the figures obtained at the beginning and end of anaerobiosis appears to make it more probable, however, that glucose is not being utilised during this period.

Levene and Meyer [1913] showed that chopped kidney tissue *in vitro* was capable of turning glucose into lactic acid, an observation which has also been recorded by Warburg, Posener and Negelein [1924]. The latter writers found, however, that the rate of glycolysis was greater anaerobically than aerobically, a fact which does not at first sight accord with the findings recorded above, but it is possible that different phases of carbohydrate utilisation have been studied in the two cases.

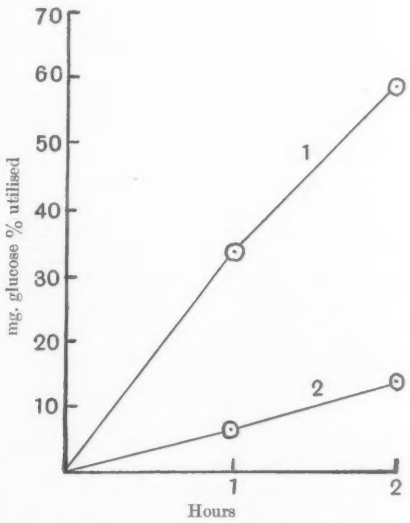

Fig. 2. The influence of cyanide.
Curve 1. Normal glucose utilisation.
Curve 2. Glucose utilisation in the presence of KCN *M*/250.

A few preliminary experiments have shown that the disappearing glucose may be converted into a polysaccharide[1]. In addition, it has been found that the contents of the reaction tubes are more acid at the end of the experiments than at the beginning, irrespective of whether glucose has been broken down

[1] Note added August 3rd. More recent experiments have shown that it is improbable that glucose is changed to polysaccharides by kidney tissue *in vitro*. J. T. I.

or not. This fact would make it appear more probable that glucose was taking part in the synthetic phase of the glycogen $\rightleftharpoons$ lactic acid cycle as found by Meyerhof, Lohmann and Meier [1925], particularly as Meyerhof [1926, 2] has shown that glucose is probably not an intermediate in the production of lactic acid from glycogen in muscle. Further work is being done to determine the fate of the glucose which is utilised.

The method of utilisation of glucose in the kidney appears, however, to differ fundamentally from that in the blood corpuscle, since the latter is not inhibited by cyanide [Irving, 1926].

The site of glucose utilisation.

A series of experiments was undertaken with a view to finding the nature of the agency which activated glucose. Attempts were first made to isolate a "glycolytic" enzyme by extraction, employing a method similar to that used by Meyerhof [1926, 1].

The extracts were usually made by grinding the chopped cortex with isotonic phosphate buffer (p_H 7·8) at $-3°$. Both the whole mass and the supernatant fluid after centrifuging off the macerated tissue were quite inactive when incubated at 38°. In addition, at the end of the ordinary aerobic experiments described in the previous section, the phosphate buffer, after removal of the kidney tissue, was not found to utilise glucose, so that the enzyme did not appear to be able to diffuse out of the cells. Finally, kidney tissue which had been previously frozen solid for a short period, and then thawed, was found to have completely lost its activity, an observation which Laquer [1922] has also made for muscle.

Alders, Chiari and Laszlo [1927] have recently stated that a glycolytic enzyme can be prepared from several tissues by incubating them in glucose-Ringer solution for 1 hour, and then filtering through a Chamberland candle. The filtrate possesses the power of degrading glucose. The present writer has not succeeded as yet in confirming these experiments, but the method of analysis employed by these authors appears to include other substances besides glucose.

It would thus appear that if an enzyme were responsible for the changes observed, it would be much less stable than that described by Meyerhof [1926, 1]. Further experiments were carried out in order to see if an active surface was involved. Several homologous alcohols, methyl, ethyl, propyl, and *iso*propyl were employed. The minimum inhibiting concentrations were investigated in each case, and were found to be very sharp, save in the case of *iso*propyl alcohol, which for some unknown reason did not give such good results as the others. These concentrations were:

Methyl alcohol...	9·9 %
Ethyl alcohol	7·8
Propyl alcohol...	2·9
*iso*Propyl alcohol	1·5

The ratio between these amounts is consonant with a surface action theory. Typical results are shown in Table III.

Table III.

Alcohol %	mg. glucose % utilised in 2 hours		
	Propyl	Ethyl	Methyl
1·5	39	—	—
2·0	9	—	—
2·4	7	—	—
2·9	0	—	—
3·4	0	—	—
3·8	—	14	—
5·7	—	—	23
6·5	—	10	—
7·1	—	18	—
7·4	—	7	22
7·8	—	0	—
8·3	—	0	—
9·1	—	—	9
9·9	—	—	0
10·7	—	—	0

In addition, reversibility of the inhibiting action was demonstrated during the early stages of the experiments. Typical results are shown in Table IV.

Table IV.

In each experiment sample no. 1 was placed in contact with alcohol for the stated period, the kidney tissue was washed in a large excess of saline and then incubated in glucose-buffer solution. Sample no. 2 was incubated in glucose-buffer with the same concentration of alcohol as a control.

Exp. no.	Hours	Sample no. 1		Sample no. 2		Conditions
		Glucose %	Glucose utilised %	Glucose %	Glucose utilised %	
1	0	0·157	—	0·134	—	Propyl alcohol
	2	0·110	0·047	0·136	0	2·9 %. 10 mins.
2	0	0·141	—	0·110	—	Ethyl alcohol
	2	0·090	0·051	0·120	0	7·8 %. 5 mins.
3	0	0·163	—	0·141	—	Methyl alcohol
	2	0·127	0·036	0·141	0	9·9 %. 10 mins.

These experiments thus show that an active surface is probably one of the main factors in the utilisation of glucose by the kidney. Whether the surface acts in the manner pictured by Quastel [1926], or whether the responsible enzyme is adsorbed on some cellular interface, it is at present impossible to say. From the facts adduced above, however, it would appear that, as with the blood corpuscle, the integrity of the cell is essential for the utilisation of glucose.

SUMMARY.

1. Kidney tissue *in vitro* utilises glucose aerobically at a rate which is probably linear. *In vacuo*, or in the presence of KCN, the utilisation of glucose is markedly inhibited, or stopped completely.

2. The integrity of the kidney cell is essential for the utilisation of glucose. It is probable that an active surface is involved.

The writer is indebted to Prof. R. A. Peters for his continued advice and interest in this work.

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CXXI. VITAMINS A AND D OF SPINACH.

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Recent work on the differentiation of vitamins A and D has emphasised the need of a vitamin D-free source of vitamin A. This has been chiefly sought for in fresh spinach leaves, which have been shown by many investigators to contain considerable amounts of vitamin A. However, in spite of much work on the problem, the possible presence of vitamin D in spinach leaves still appears to be a matter of debate.

The first investigators to use spinach for the supply of fat-soluble vitamins appear to have been Steenbock and Gross [1920], who found that the dried leaves when fed to rats at the level of 5 % of the diet as the sole source of vitamin A, produced continued but subnormal growth over periods of 6 or 7 months. Osborne and Mendel [1920], employing an ether extract of dried spinach leaves, obtained adequate growth and cure of xerophthalmia with doses of 66 mg., equivalent to about 10 to 20 g. of fresh leaves. McClendon and Sedgwick [1921] secured satisfactory growth in rats with dried spinach administered in amounts equivalent to about 5 or 6 g. of the fresh leaves, and also found it of benefit for children. In these investigations vitamin A was not distinguished from vitamin D.

After the differentiation of the fat-soluble vitamins attention was turned to the possible antirachitic properties of spinach. McClendon and Shuck [1923], using dried spinach in amounts equivalent to about 8 or 9 g. of the fresh leaves, observed no antirachitic action. Zucker and Barnett [1923], who employed an acetone-ether-alcohol extract of the dried leaves, found its growth-promoting potency comparable with that of cod-liver oil, but no significant amount of vitamin D was detected. The amount fed, however, was not stated. Goldblatt and Zilva [1923], feeding the fresh leaves, obtained some growth with less than 0·25 g., but no increase in calcification in rats on a rachitogenic diet with doses up to 3 g., as much as it was found practicable to administer, and concluded that "there is a great disparity between the growth-promoting and the antirachitic potency of spinach."

The next step came when it was discovered that the antirachitic vitamin could be formed by the action of ultra-violet light, either that naturally present in sunlight, or artificially produced. Hess and Weinstock [1924] found

no appreciable amount of vitamin D in spinach or other green leaves, but were able to produce it by irradiation. Following up this idea, Chick and Roscoe [1926] showed that fresh spinach might contain slight amounts of vitamin D if collected in the summer, while Boas [1926] concluded that winter spinach contains an amount of vitamin D which is negligible compared with its content of vitamin A. Later, an attempt was made by Roscoe [1927] to increase the content of vitamin D by further exposure of the leaves to the ultra-violet rays in sunlight, but the results obtained were not conclusive. Thinking that this might be due to rats not eating sufficient greenstuff, Roscoe also tested "sunned" spinach on rabbits. It could not be shown conclusively that exposure to sunlight increased the content of vitamin D, but Roscoe succeeded in demonstrating that fresh spinach contains more of this vitamin than had previously been suspected.

While the substitution of the rabbit for the rat as an experimental animal in testing for vitamin D enables larger proportions of spinach to be included in the diet, our opinion was that the use of this modified technique involves new difficulties. First, the digestive and assimilative processes in a herbivorous animal are different from those in an omnivorous animal, the gastro-intestinal p_H varying considerably and being closely connected with the rôle of the vitamin [Redman, Willimott and Wokes, 1927]. Secondly, from the recent work of Mellanby and Killick [1926], it seems probable that the optimum conditions for calcium absorption in the rat differ from those in the rabbit. Thirdly, the administration of large doses of fresh spinach leaves necessarily involves the ingestion of considerable quantities of calcium and phosphorus, with consequent alteration of the calcium/phosphorus ratio. (Fresh spinach contains 0·09 % CaO and 0·13 % P_2O_5 according to Sherman [1911].) For these reasons we decided to employ a practically mineral-free extract of dried spinach leaves which could be administered to rats in large doses without upsetting the calcium/phosphorus ratio.

Experimental.

Preparation of the spinach extract.

7167 g. of fresh English spring spinach (picked at the beginning of May, 1926) were sorted, spread out on sheets of paper and dried at 18° for 2 days. The resulting product was a light green material weighing 726 g. This was first broken up by rubbing between the hands, the midribs and stalks were then removed, and the residue was ground in a mortar and sieved through a no. 10 sieve. The product was a light green powder weighing 640 g. This material was shaken with dry ether at room temperature for 3-hour periods, three successive lots of about 3 litres each being used. The first extract was dark green leaving a residue coloured light yellow-green. The marc was pressed on a Büchner funnel by hand so as to remove as much of the solvent as possible. This pressed marc on treatment with a second lot of ether gave a

much less darkly coloured extract, while the third extraction yielded a product only slightly coloured. The residue was then extracted with two successive lots of dry acetone, but little colour was obtained. The whole of the ether and acetone extracts were filtered, combined, and the solvent removed as rapidly as possible *in vacuo*. The resulting product was a dark green pasty mass, weighing 32·5 g. and representing 0·45 % of the fresh leaves. This was put aside in a desiccator protected from light, over sulphuric acid, and after a few days small red crystals were observed, probably of carotene and xanthophyll. The extract was stored in a desiccator filled with nitrogen and protected from light while in use.

Tests applied to the extract showed it to be free from oxidising enzymes (oxygenase and peroxidase). The ash was about 4·5 %. Since the fresh leaves contain about 2 % of ash [Plimmer, 1921], and the extract was concentrated over 200 times, only about 1 % of the mineral matter in the fresh leaves had passed into the extract. On taking up in light petroleum a few grams of the extract, decolorising with norite, filtering and evaporating, a colourless oily residue was obtained which gave transient blue colours with both arsenic trichloride and antimony trichloride [Willimott and Wokes, 1927]. Takahashi [Takahashi *et al.* 1925] using a vitamin A concentrate prepared both from cod-liver oil and from spinach, obtained transient blue colours with concentrated sulphuric acid, but we have not been able to trace any record of these colours, supposedly due to the vitamin, having been previously obtained with the former two reagents on a spinach extract.

Feeding experiments using high fat diets.

In a previous communication [Redman, Willimott and Wokes, 1927] it was pointed out that in tests for vitamin D two types of diet have been principally used by different investigators, viz. a high fat diet containing 15 to 17 % of fat, and a low fat diet containing less than 3 %. The high fat diet resembles the natural diet of the infant the more closely both in fat content and in its calcium/phosphorus ratio. It has been largely used by English workers, who have adopted bone changes as their chief criteria of antirachitic potency, but so far as we are aware, no results have yet been published of the faecal p_H of rats on high fat diets free from vitamin D. In view of the findings of Zucker and Barnett [1923] and the more recent work of Jephcott and Bacharach [1926] that the faecal p_H in the rat can be used to test for vitamin D, we thought it of interest to feed to rats a high fat diet in which the spinach extract previously described served as sole source of vitamin A, and vitamin D if any. The faecal p_H was observed as well as the bone changes more commonly employed as criteria for vitamin D.

Standard albino rats of Wistar Institute ancestry were placed on the basal diet usual in this laboratory containing 15 % fat, vitamin B being supplied by an extract of yeast and vitamin C by lemon juice. The spinach extract was administered at first by hand in advance of the basal ration but as the animals

consumed it readily without objection, the required dose was afterwards uniformly mixed in with the basal diet. By following the food intake, it was possible to give an amount of the extract such that each animal received on the average about 25 mg. Much larger amounts are consumed by rats without difficulty.

Table I. *Spinach extract used with a high fat diet as sole source of vitamins A and D.*

Group	Sex	No. of rats	Supply of vitamins	Daily dose (mg.)	Average weekly weights (g.)	Rickets: Microscopic	Rickets: A.R. ratio	Xerophthalmia
Experimental 1	♂	3	Spinach extract	25	55 72 79 91 102 109 126 130 133 141	Very slight	1·15	Absent
	♀	3	Spinach extract	25	48 60 74 82 87 95 98 100 101 106	Very slight	1·21	Absent
Positive control	♂	3	Cod-liver oil	50	56 72 94 119 141 164 178 194 210 221	Absent	1·25	Absent
	♀	3	Cod-liver oil	50	48 62 82 96 107 118 129 138 142 143	Absent	1·29	Absent
Negative control	♂	2	Vitamin D as direct irradiation of the animals	—	55 59 72 85 88 87 84 86 75	Absent	—	Present in two animals
	♀	3		—	53 55 68 80 81 83 83 76 74	Absent	—	

Group	Sex	No. of rats	Supply of vitamins	Daily dose (mg.)	Average faecal p_H								
Experimental 1	♂ & ♀	6	—	—	6·8	7·0	7·1	7·4	7·4	7·4	—	—	—
					7·5	7·5	7·5						
Negative control	♂ & ♀	5	—	—	6·8	7·0	6·9	6·8	6·7	6·8	—	—	—
					6·7	6·8							

On account of the different rates of growth in the two sexes, separate records were made of the average weights of males and females, in each of the three groups. The groups were chosen so as to be well balanced in regard to age, weight, sex and litter origin, each containing originally three males and three females, but in the negative control group one male died just after the beginning of the experiment from some unknown cause, and was not included in the average data. Of the female negative controls, one died in the third week and a second in the eighth week, and their weights are included in the average data. The surviving female, and the two males, were chloroformed and autopsied at the end of 8 weeks, when vitamin A deficiency was pronounced.

The experimental results are summarised in Table I. It will be seen that 25 mg. of the spinach extract, equivalent to about 6 g. of the fresh leaves, secured freedom from xerophthalmia and steady though subnormal growth in all the six experimental animals over a period of 10 weeks. It is noteworthy that Chick and Roscoe [1926], using a similar high fat diet, and fresh spring spinach, did not succeed in entirely preventing xerophthalmia with doses reaching in one case as high as 2·6 g., although the growth obtained was rather better.

In regard to rickets, the bone changes in our experiments were not conclusive. Autopsies on the animals revealed very slight rickets in several but not in all cases, which is in agreement with the findings of Chick and Roscoe. The average A.R. ratio was only very slightly lower than normal. On the other hand, the faecal p_H became alkaline and, while fluctuating somewhat, remained on the average faintly alkaline throughout the experiment. From this it would appear that an alkaline faecal reaction is specific to absence of vitamin D, whether rickets develops or not, but the matter requires further investigation. At the end of the experiment, vitamin D was supplied to the remaining animals in two ways, either as cod-liver oil or by direct irradiation of the animals, the diet not being altered, and in each case the faecal reaction changed within a few days to definitely acid (p_H below 6·8).

Feeding experiments using low fat diets.

The low fat, vitamin D-free diet has been used mainly by American investigators, who find that it produces within a few weeks a definite rachitic condition recognisable either by bone changes or by an alkaline faecal p_H. Since our previous experiments with the high fat diet had shown that a 25 mg. dose of the spinach extract was apparently too low to supply sufficient vitamin D, we decided to administer a much larger dose of 100 mg., equivalent to over 20 g. of fresh leaves, or more than twice as much as had previously been given by other workers testing for vitamin D in spinach.

Four standard albino rats were put on Zucker's modified low fat diet, consisting of patent flour 85, powdered egg-albumin 10, calcium lactate 2·8, sodium chloride 2, ferric citrate 0·2 parts by weight, with 2 g. of fresh spinach leaves per animal per day. The faecal p_H was ascertained daily, both colorimetrically and electrometrically, employing the methods previously described [Redman, Willimott and Wokes, 1927]. The results are shown in Fig. 1, in which the curve is built up from the average p_H of the total faeces collected from the group of animals. The individual variations between different animals, and between samples obtained from the same animal at different times on the same day, were investigated by means of the capillary electrode, and it was found that the greatest variation was 0·3 p_H, but on the average the results agreed to within 0·2 or less.

After 9 days on this diet, the faecal reaction had become definitely alkaline (average p_H 7·7). When it had remained alkaline for several days, each animal was given separately a daily dose of about 100 mg. of the spinach extract by hand, in advance of the basal ration, the supply of fresh spinach leaves then being discontinued. This was readily consumed without exception, and the food intake rose slightly from about 9 g. per head per day before dosing to about 11 g. after dosing. Xerophthalmia was absent throughout the experiment. A slight drop in alkalinity resulted on administration of the 100 mg. doses of extract, but at the end of another 12 days the faeces were

still alkaline (average p_H 7·3, Fig. 1). On reducing the dose of extract to 50 mg., the faecal p_H became slightly more alkaline (average p_H 7·5). After the reaction had remained at this level for another 10 days, cod-liver oil was administered in doses of 0·1 g., and the change of average faecal p_H to below 6·8 within a week showed that the animals were reacting normally to the administration of vitamin D. The animals were immediately chloroformed and autopsied. Definite rickets was observed, and the A.R. ratio was found to range between 0·7 and 0·8. It would appear, therefore, that the spinach extract, when fed at a level supplying adequate amounts of vitamin A, does not contain significant amounts of vitamin D.

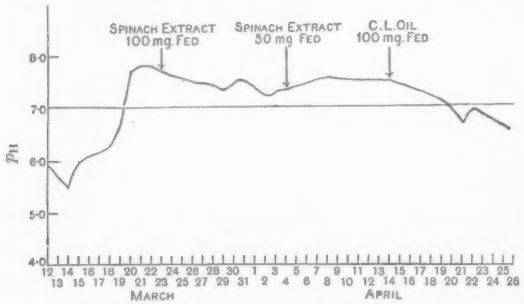

Fig. 1. Effect of feeding spinach extract to rats on Zucker's low fat rachitogenic diet.

Table II. *Summary of results obtained by various workers for content of vitamins A and D in spinach leaves.*

Reference	Type of diet	Form in which spinach was fed	Approx. equivalent in fresh leaves (g.)	Growth	Xerophthalmia	Rickets
Steenbock and Gross [1920]	—	Dried leaves	5–6	Steady but subnormal	Absent	—
Osborne and Mendel [1920]	High fat	Ether extract	15–20	Adequate	Cured	—
McClendon and Shuck [1923]	Low fat	Dried leaves	8–10	Adequate	Cured	No antirachitic property
Chick and Roscoe [1926]	High fat	Fresh leaves	0·5–2·6	Adequate	Very slight	Slight (? traces of vitamin D present)
Roscoe [1927]	Low fat	Fresh leaves	1·5	—	—	Severe, but small amount of vitamin D present
Willimott and Wokes [1927]	High fat	Ether-acetone extract	5	Steady but subnormal	Absent	Very slight
	Low fat	Ether-acetone extract	10	Adequate	Absent	Definite
	Low fat	Ether-acetone extract	20	Adequate	Absent	Some vitamin D present

Note. In calculating the approximate equivalent in fresh leaves of the doses of dried leaves, the fresh leaves have been assumed to contain about 92 % of moisture [Plimmer, 1921].

Discussion.

In many previous investigations on spinach as a possible source of vitamins A and D, sufficient attention does not appear to have been paid to various factors which might affect the content of these two vitamins. Amongst these factors are exposure of the spinach to ultra-violet light, either artificially produced or naturally present in sunlight, to the action of oxidising enzymes known, from the work of Ciamician and Ravenna [1919], to be present in spinach leaves, or to heat and oxidation during the drying processes. Moreover, the variations in vitamin content of the fresh leaves, according to the amount of sunlight they have received, and the difficulty of feeding them regularly to rats in large doses, have thrown serious obstacles in the way of obtaining satisfactory quantitative data. In Table II are summarised results obtained by different workers, which show considerable variations in their estimate of the content of fat-soluble vitamins in spinach leaves. These differences are probably accounted for by the factors indicated above and possibly also by factors still unknown.

In our opinion, these varying factors can best be controlled by the use of an acetone-ether extract of the dried leaves which, when carefully prepared under given conditions, is a potent and stable source of vitamin A practically free from calcium and other mineral matter and from oxidising enzymes. The employment of such an extract has allowed the feeding of much larger doses of spinach than hitherto, and it has been found that the extract when fed at a level supplying ample quantities of vitamin A, does not contain more than traces of vitamin D. However, the fact that vitamin D may be present in these small amounts, which can be greatly increased by exposure to the action of the same agent (ultra-violet light) which under certain conditions appears to be responsible for the destruction of vitamin A, raises the interesting question of a possible balance between these two vitamins, a problem for further investigation.

Summary.

1. An acetone-ether extract of dried spring spinach has been prepared more than 200 times as concentrated as the fresh leaves, and practically free from mineral matter and oxidising enzymes.

2. The extract was a potent and stable source of vitamin A in that 25 mg. daily doses secured complete freedom from xerophthalmia and induced satisfactory though slightly subnormal growth in rats.

3. The extract, when fed at this level, does not appear to contain significant amounts of vitamin D.

We are indebted to Sir F. G. Hopkins and to Prof. J. M. Beattie for their interest and support, and for kindly examining some of the experimental animals.

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CXXII. ON THE EARLY STAGES OF MICRO-BIOLOGICAL DECAY AND HUMIFICATION OF VEGETABLE TISSUES.

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(*Received June 4th, 1927.*)

In a previous communication [Thaysen, Bakes and Bunker, 1925] it was shown that the humification of vegetable tissues in peat bogs affects both the carbohydrate and the lignin constituents of the tissues. It was possible to demonstrate that the humus recoverable from peat is composed of two definite substances, one showing the characteristics of humus obtainable from carbohydrates after treatment with acid, and another functioning on chlorination as humus prepared from quinol or lignin.

A sample of humus behaving on chlorination as a typical carbohydrate humus was obtained also from a piece of ancient Egyptian linen cloth from the tomb of Tut-ankh-amen. The amount of humus collected from this source was of the order to justify the conclusion that it had been formed from the cellulose of the linen fibres through "ageing."

This was thought to indicate that the cellulose of vegetable tissues decaying in a peat bog might suffer a similar conversion through "ageing" and that the fraction of peat humus which had been shown to be identical with a carbohydrate humus in its behaviour on chlorination had been derived from cellulose.

In the course of certain investigations carried out on behalf of the Fuel Research Board, an opportunity arose of following the progress of decay of vegetable tissues, and it was thought of interest to ascertain the quantitative relationship of the two fractions previously recognised as present in natural humus, at various stages of the progress of humification, and to establish, if possible, the constituent of the vegetable tissues which served as raw material for the carbohydrate fraction.

The material which was subjected to humification was chopped oat straw. To secure humification its moisture content was increased to 50 % by addition of the requisite amount of water. In this water had been previously dissolved sufficient ammonium carbonate to raise the nitrogen content of the damped straw to 2 %.

After wetting, the straw was packed in boxes sufficiently large to hold 20 lbs. each. The sides of these boxes were made of wire netting to facilitate access of air to the material. The boxes were incubated at 22–25°. An inoculant

was not added to the straw as previous experiments had shown that the microflora of oat straw is capable of producing the high temperatures which appear to be, if not essential, at least highly favourable for the humification to proceed rapidly.

Any loss of moisture suffered by the straw heaps was made good every third to fourth day.

The temperature of the packed straw rose rapidly and had reached a figure of 32° on the fifth day after incubation. On the seventh day a thermometer inserted in the heaps registered 49·6°. This temperature remained unchanged during the following 6 days and then gradually declined to 35°, a figure which was reached on the fortieth day. The lowest temperature recorded was 27·5° after 62 days' incubation of the straw.

A detailed bacteriological analysis of the decaying straw was carried out on the sixth day when the temperature of the straw had reached 46°. The number of micro-organisms developing on ordinary agar at 37° at this time amounted to 4900 millions per g. of damp straw, while 1800 millions developed on wort agar from the same quantity of straw. The microflora was very varied, though consisting essentially of aerobic bacilli of the groups of *Bac. mesentericus*, *Bac. dendroides* and *Bac. mycoides*. Various types of *Micrococcus* were observed and in addition *Bact. Zopfii*, *Bact. fluorescens liquefaciens*, *Bact. prodigiosum*, *Bact. herbicola aureum*, a number of actinomycetes and species of *Aspergillus* and *Penicillium*. The daily microscopic examination of the decaying straw revealed, as had already been observed by Miehe [1911], that the non-spore-forming types made their appearance first and were followed by various spore-formers when the temperatures had risen to a height.

On the twenty-fifth day an extensive growth of a species of *Coprinus* developed on the straw, the mycelium of which spread throughout the mass of the straw in the course of 2 days. Subsequently numerous fructifications of this fungus appeared on the surface of the straw.

By the forty-fourth day the microbiological activity appeared to have greatly diminished and most of the heat-resisting types could be observed only in the form of their spores. By inoculating dilutions of a suspension of the material into medium containing xylan and cellulose it was established that micro-organisms capable of decomposing both hemicelluloses and cellulose were present in the decaying straw.

There must have been more than 100 millions of hemicellulose-fermenting bacteria present per g. of straw since a dilution of 1 in 100 million of the straw showed growth in the xylan medium after 4 days' incubation at 37°. The types developing were not the aerobic spore-bearing rods capable of fermenting hemicelluloses (*Bac. mesentericus*, etc.).

The media containing cellulose which had been inoculated with 1×10^{-6} g. of decaying straw showed growth and destruction of the cellulose after incubation at 65°. Incubated at 37°, growth and cellulose decomposition did not occur from dilutions higher than 0·001 g. of straw. This observation

indicates that a destruction of cellulose in the fermenting straw proceeded most vigorously from about the sixth to the twenty-ninth day, while the temperature of the straw remained above 37°.

The experiments by which it was hoped to throw light on the relative importance of the hemicelluloses and the cellulose for the formation of humus in decaying straw included the following determinations at repeated intervals.

(1) *The alkali solubility of the straw.* Here 4 g. of dried decaying straw were extracted under a reflux condenser with 200 cc. of 3 % sodium hydroxide. The residue was collected on weighed filter paper, washed, dried and weighed.

(2) *Hemicelluloses* (pentosans, estimated by Tollen's method [de Chalmot and Tollens, 1891; Günther *et al.*, 1890, 1891]).

(3) The *cellulose* (estimated by the method of Cross and Bevan [1903]).

(4) *Humic substances.* The filtrate from the alkali solubility determination was acidified with 30 cc. of concentrated hydrochloric acid, the precipitate left overnight and filtered through weighed paper, washed, dried and weighed.

The results of these determinations are compiled in Table I.

Table I*.

Sample	Days of fermentation before removal of sample	Temperature of heap on removal of sample ° C.	Alkali solubility of sample %	Hemicellulose content of sample %	Cellulose content of sample %	Humus content of sample† %
Original straw	—	25	48·5	31·3	39·86	22·4
Decaying straw	14	49·6	49·53	25·7	39·86	23·4
,,	28	40·5	56·66	18·97	35·94	26·26
,,	64	27·5	64·95	14·44	27·94	30·9
,,	82	27·5	67·84	11·07	23·78	31·55
,,	102	27·5	71·14	10·1	16·38	32·8
,,	146	27·5	70·6	7·93	—	32·3

* In interpreting the results of Table I it should be kept in mind that no attempt could be made to adjust the figures given for any losses suffered by the straw through the evolution of gases. The recorded data represent the percentages found in the sample as available on the day of collecting and after drying. The figures therefore under rather than over-estimate any changes which have occurred.

† It has been mentioned already that the figures given in this column were based on the weight of the precipitate collected on acidifying the extracts obtained by boiling the straw sample with a 3 % sodium hydroxide solution. They do not therefore give a true picture of the humus content of the samples since other substances, notably the hemicelluloses and lignin constituents, are brought into solution and reprecipitated under the conditions of experiment.

The most rapid increase in humus production took place between the first and the sixty-fourth day of fermentation, at a time when the hemicellulose content of the fermenting straw had decreased from 31·3 to 14·44 % and the cellulose from 39·86 to 27·94 %.

With a view to ascertaining the relative proportion of carbohydrate and lignin fractions in the humus during this and the immediately succeeding period, samples of humus were collected at various stages and subjected to chlorination by Eller's technique [Eller *et al.*, 1923].

The separation of the chlorinated fractions by extraction with ether showed, as indicated below (Table II), that the proportions of the two fractions varied appreciably with the progress of the humification.

Table II.

Days' fermentation before collection of humus from straw	Carbohydrate fraction in humus (%)	Lignin fraction in humus (%)
28	28·0	72·0
49	40·7	59·3
82	48·6	51·4
102	38·4	61·6
122	36·82	63·18

During the early period of humification of the oat-straw there was thus a steady rise in the percentage of the carbohydrate fraction, a rise which reached its maximum after about 80 to 85 days' fermentation. The extent of the subsequent decline in the percentage of this fraction could not be followed beyond the 122nd day of fermentation. However, it is interesting to note in this connection that a sample of silage which had remained packed in a silo for 3 years showed a humus content of 31·0 %, composed of 38·6 % carbohydrate humus and 61·4 % of lignin humus. And in a sample of peat humus examined previously [Thaysen *et al.*, 1925] and estimated to have taken 40 years to form, the carbohydrate fraction still amounted to 39·28 % of the total.

Whether the observed decrease in the lignin fraction of the humus was due to an actual destruction of lignin during decay or to an increase in the carbohydrate fraction beyond that indicated in Table II may be deduced from a consideration of the figures recorded in this and in Table I.

On the twenty-eighth day of fermentation there were present in 100 g. of the decaying straw 26·26 g. of humus containing approximately 18·9 g. of lignin fraction. On the eighty-second day of fermentation the humus of 100 g. of decaying straw had increased to 31·55 g. containing 16·22 g. of lignin fraction. During the whole of this period of intensive humification therefore the lignin fraction had remained practically constant, while the carbohydrate fraction had doubled—from 7·3 g. in the humus of straw after 28 days' fermentation to 15·33 g. in the humus of straw after 82 days.

At the time of the presence of these 15·33 g. of carbohydrate humus in the decaying straw a loss of 20·33 % of hemicelluloses and of 16·08 % of cellulose had been suffered by the original straw. With no information in existence on the efficiency with which carbohydrates are humified under natural conditions it was impossible to say whether the losses of hemicelluloses (pentosans) or those of cellulose were sufficient to account for the formation of these 15·33 g. of carbohydrate humus.

Light can be thrown on this question, however, by examining the position at an earlier stage of the decay, after 28 days of fermentation.

In the sample of straw taken after 28 days there were present in 100 g. of straw 7·36 g. of carbohydrate humus. At this stage the straw had lost 12·33 % of hemicelluloses (pentosans), an amount which conceivably might have been sufficient to yield 7·36 g. of humus. It is unfortunate that evidence cannot be brought forward in support of this. During the same period of fermentation the straw had lost only 3·92 % of cellulose, an amount which even under the most favourable conditions could not have yielded 7·36 g. of humus per 100 g. of straw. It is evident therefore, that, whether responsible for the production of the whole of the carbohydrate humus or not, the hemicelluloses (pentosans) must have taken part in its formation. This conclusion is in agreement with the views of Stoklasa [1898] and of Omelianski [1905] who regard pentosans as particularly important for the increase in the fertility of the soil.

The interesting question of the fate of the carbohydrate fraction of natural humic substances during the process of coal formation unfortunately did not come within the scope of the present enquiry. In a few preliminary experiments it was possible to show that the regenerated humus of three samples of bituminous coal gave chlorine derivatives containing on an average 35·36 % of chlorine. These derivatives, which were of a deeper orange appearance than those of humus derived from recently decayed plant tissues, were entirely soluble in ether. They did not therefore contain typical carbohydrate fractions. The fact that their chlorine content was of the order of that of a mixture of carbohydrate and of lignin humus may perhaps be taken as an indication that the solubility of the whole of the derivatives in ether cannot be regarded as sufficient support for the conclusion that a carbohydrate fraction was absent in these cases.

The regeneration of the humus of the three samples of bituminous coal was carried out by a method recommended by Dr Francis of the Safety in Mines Research Board, Sheffield, in a private communication.

One of the coal samples labelled "Ellistown Main Brieze" was received from the Fuel Research Station, East Greenwich, the two others were samples of ordinary household coal obtained from local coal merchants.

The regenerated humus from the Ellistown sample amounted to 69·44 % of the coal. The corresponding figures for the two other coals were somewhat higher owing to the lower ash content of these coals.

While the above investigation was in progress, a sample of mummified tissues, consisting of a human vertebra with adhering muscular tissues was received by Mr H. J. Bunker of this laboratory from Mr A. Lucas, formerly Director of the Chemical Department of the Egyptian Government, and an opportunity was taken to establish the nature of, and to examine the changes which had occurred in, these tissues.

The material dated from about 200 B.C. and was stated to have become completely humified. On examination its muscular tissues were found to possess an alkali solubility of 82·1 %, while its humus content amounted to 33·8 %.

On chlorinating, the humus took up 22·81 % of chlorine. The chlorine derivative was very similar in appearance to the chlorine derivative of the carbohydrate fraction of vegetable humus. It resembled it also in being almost insoluble in ether.

An ultimate analysis showed the chlorinated humus from mummified muscular tissues to contain 14·16 % of C; 3·62 % of H and 22·81 % of Cl corresponding to a formula of $C_{21}H_{21}O_{13}Cl_4$, the formula of the chlorine component of the carbohydrate fraction of vegetable humus.

Summary.

The early stages of humification of plant tissues (oat-straw) as induced by micro-organisms have been studied. It has been shown that the hemicelluloses (pentosans) of the raw material are at least partly responsible for the appearance of the carbohydrate fraction of the humus.

In three samples of bituminous coal no definite proof could be obtained of the presence of a carbohydrate fraction in the regenerated humus, though the percentage of halogen in the chlorine derivatives of these humus samples was of the order characteristic of a mixture of carbohydrate and lignin humus.

The muscular tissues adhering to a mummified human vertebra dating from 200 B.C. had been converted into a humus with the properties of a typical carbohydrate humus.

The above experiments arose out of work carried out on behalf of the Fuel Research Board of the Department of Scientific and Industrial Research. The authors desire to express their thanks to the Department of Scientific and Industrial Research and to the Admiralty for permission to publish the results obtained.

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CXXIII. THE RELATION OF THE GROWTH OF CERTAIN MICRO-ORGANISMS TO THE COMPOSITION OF THE MEDIUM.

I. THE SYNTHETIC CULTURE MEDIUM.

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(*Received June 11th, 1927.*)

This investigation had as its main object the study of the influence of the ill-defined growth-promoting factor known as bios on the development of unicellular organisms in artificial media. In the early stages it became apparent that no appreciable progress could be achieved until the other essential requirements had been more clearly defined. In particular, this referred to their needs for inorganic salts and the most suitable sources of carbon compounds from which they could derive the necessary energy for their metabolic processes.

It is a curious fact that, in spite of a very large amount of work reported in the literature, the question of the most suitable composition of the artificial medium in which a particular organism can be grown is undecided. This is particularly true of the inorganic composition of the culture fluid, and there seems to be a general impression that the requirements of yeast and bacteria for mineral salts are moderately elastic [Pasteur, 1860; Liebig, 1871; Wildiers, 1901; Windisch, 1902; Pringsheim, 1906; Osborne and Wakeman, 1919; Miller, 1921]. From the experiments reported below it must be concluded that only within a very narrow range of concentration is maximum growth obtained.

Experimental.

Preliminary experiments showed that all the organisms tried would grow on a medium consisting of pure glucose, certain inorganic salts, and water when these were added to clean flasks, sterilised on three successive days, inoculated, and incubated. Consequently it was decided to study in detail the basal requirements of three organisms and find how far (1) the inorganic salt content, and (2) the source of carbon could be varied.

The organisms used were *Sarcina aurantiaca*, *Streptothrix corallinus*, and a white *Streptothrix* as supplied by the National Collection of Type Cultures.

The strains to be used were grown on the surface of broth agar, and carefully plated to make sure that they were pure cultures. Inoculations were always made by suspending some of the dry surface growth in water, using a platinum loop. To avoid acclimatisation effects a fresh culture was used after every four or five subcultures had been made. All water used for making up the media or for the suspensions was prepared from tap water, by distilling, boiling for 3 hours with alkaline permanganate, and redistilling, only glass or silica vessels being used throughout.

The chemicals used were either Kahlbaum's chemicals "Pure for Analysis" or "Analytical Reagents" from the British Drug Houses. The NaCl and $MgSO_4$, $7H_2O$ were further recrystallised. The glucose employed was the "Puriss" variety from the British Drug Houses, but it was found to need four recrystallisations from 80, 75, 70 and 65 % of alcohol before it was rendered free from detectable traces of impurities having a definite stimulating action on growth.

Optimum hydrogen ion concentration. The limiting p_H was found for each organism by inoculating a series of plain broth media tubes and plates of different p_H before attempting to make up a suitable synthetic medium. The approximate limits were found by grading the series by means of colorimetric methods; later a more accurate adjustment was obtained by readings with the glass electrode [Kerridge, 1925]. A series of flasks for each organism was prepared, the contents of the flasks differing from each other by a p_H of 0·02 over the range 4·50 to 10·00. The results were as follows:

Organisms	Lowest limit at which appreciable growth occurred p_H	Maximum growth p_H	Highest limit p_H
1. *S. aurantiaca*	5·30	7·15	9·43
2. *Streptothrix corallinus*	6·02	7·60	9·35
3. White *Streptothrix*	6·02	7·60	9·35

Later these values were checked when a satisfactory artificial medium had been elaborated and were found to be substantially correct. Meanwhile it was decided to work at a value of $p_H = 7·4$ throughout.

The inorganic salt content. Having established the optimum hydrogen ion concentration for growth, a series of experiments was carried out in which varying quantities of inorganic salts were added to a medium containing 1 % of dextrose.

Table I.

Medium: Glucose 1 %
KH_2PO_4 0·1 %
K_2HPO_4 to $p_H = 7·4$.

Salt	Percentage concentration											
	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*	*K*	*L*
$(NH_4)_2SO_4$	0·1	0·2	0·4	0·1	0·2	0·4	0·1	0·2	0·4	0·1	0·2	0·4
$MgSO_4$, $7H_2O$	0·02	0·02	0·02	0·05	0·05	0·05	0·07	0·07	0·07	0·09	0·09	0·09

(1) A series of flasks was arranged with 12 variations as seen in Table I. Six flasks were prepared for each group in an attempt to eliminate errors in inoculation, etc.

Growth at the end of 7 days was measured by opacity and found to be much better in cultures *E*, *F*, *H*, and *I* than in any of the others. Since in cultures *D* and *G* growth was less good than in *E* and *H* it was concluded that 0·1 % of $(NH_4)_2SO_4$ was not sufficient. It also seemed apparent that the optimum concentration of $MgSO_4$, $7H_2O$ lay between 0·05 and 0·09 %.

(2) All the ammonium-magnesium combinations in Table I were duplicated (*a*) with twice, and (*b*) with half the concentration of phosphates

$$(0{\cdot}1\ \%\ KH_2PO_4 + 0{\cdot}016\ \%\ K_2HPO_4),$$

but in neither case was the growth as good as in *E*, *F*, *H* and *I* above. It was therefore concluded that no advance could be made by altering the initial figure for phosphate concentration.

(3) The influence of the addition of varying concentrations of NaCl was next considered. A series of cultures similar to *D*, *E*, *F*, *G*, *H* and *I* was prepared, but containing in addition concentrations of NaCl varying from 0·01 % to 1 %. The cultures containing 0·05 % showed the best growth.

(4) A few preliminary experiments having indicated that traces of $Ca(NO_3)_2$ seemed to be beneficial, it was decided to set up a series containing $Ca(NO_3)_2$ over a range of concentrations from 0·001 % to 1 %. The improvement was very slight in the lower concentrations and growth was definitely retarded with concentrations above 0·075 %. The action seemed independent of concentration below 0·05 %.

(5) Innumerable examples occur in the literature of the last 20 years, some claiming, some disclaiming, the stimulating effect of traces of manganese and iron in the soil or culture medium on the growth of plant tissue. Experiments were designed in which traces of manganese salts, and ferrous and ferric salts were added to the glucose-water-inorganic salt medium described above. The substances used and the results obtained can be seen in Table II.

Table II.

Substance used	Concentration g. in 10 cc.	Increase in growth
MnO_2	1×10^{-4}	Nil
MnO_2	1×10^{-6}	,,
$MnSO_4$	1×10^{-4}	,,
$MnSO_4$	1×10^{-6}	,,
$FeSO_4$	1×10^{-4}	,,
$FeSO_4$	1×10^{-6}	,,
$FeCl_3$	1×10^{-4}	Slight
$FeCl_3$	1×10^{-6}	,,

The slight increase in growth with $FeCl_3$ was probably due to impurities, for after further purification (solution in ether, evaporation of the ether, and volatilisation of the salt) no sign of any action was detectable.

Conclusion from experiments with varied amounts of inorganic salts. From the experiments recorded above it was decided to use the following salt-mixture when studying (1) the effect of variation in the amount of glucose present, and (2) the most suitable source of carbon supply:

$(NH_4)_2SO_4$	...	...	0·30 g. in 100 cc. medium
$MgSO_4, 7H_2O$	...	...	0·07 ,, ,,
KH_2PO_4	...	...	0·10 ,, ,,
K_2HPO_4	...	...	0·016 g. (to bring p_H to 7·4)
NaCl	...	...	0·05 g. in 100 cc. medium
$Ca(NO_3)_2$	...	...	0·04 ,, ,,

Variation in the amount of glucose present. The arbitrary value of 1 % of glucose was used throughout the earlier experiments. It was decided to test this figure, using the salt concentrations tabulated above. Table III shows the results obtained over the range of concentration 0·01–10 %.

Table III.

Concentration glucose (%)	Growth
0·01	↑ Gradually diminishing
0·02	
0·03	
0·04	
0·06	
0·08	
0·10	
0·20	
0·30	
0·40	
0·50	No appreciable variation
0·60	
0·70	
0·80	
0·90	
1·00	
2·00	
3·00	
4·00	
5·00	
6·00	
7·00	
8·00	
9·00	
10·00	↓ Gradually diminishing

0·5 % of glucose was used for all later experiments.

Influence of source of carbon. Although the medium described above gave good results, it was thought of interest, in view of the number of publications on this subject, to study the value of sources of carbon other than glucose. The growth with glucose as the source of carbon is taken as standard and recorded by two crosses, *e.g.* ++.

Three crosses = increased amt. growth.

One cross = decreased amt. growth.

Table IV.

Source of carbon (0·5 % solution in salt-water medium)	Growth	Source of carbon (0·5 % solution in salt-water medium)	Growth
Glycerol ...	+ + +	Sodium pyruvate	+ +
Mannitol ...	+ + +	Fructose	+
Glucose ...	+ +	Rhamnose ...	+
Arabinose ...	+ +	Sodium acetate ...	+
Glycogen ...	+ +	„ propionate	+
Calcium lactate	+ +	„ butyrate	Trace
Sodium lactate...	+ +	„ oxalate ...	Trace
„ citrate...	+ +	„ succinate	0
		„ tartrate ...	0

The growth on glycerol and mannitol calls for comment. Although the suspension was more opaque, the rate of reproduction did not seem to be increased, but the growth was more filamentous, in fact almost vegetative. Whereas the segmentation of the *Streptothrix* usually begins about the third day, it could be delayed to the seventh or eighth day with mannitol or glycerol as the source of carbon. The effect of citrate is surprising, but the presence of mycelial threads in the culture fluid points to an effect similar possibly to that of glycerol or mannitol.

Amino-acids as source of carbon or nitrogen. The following experiments were designed to test the amino-acids (1) as a source of carbon, and (2) as a source of nitrogen for bacteria. Column 1 in Table V shows results obtained when the glucose was replaced by a 1 % solution of the various amino-acids tabulated. Column 2 gives results when the ammonium sulphate was replaced by the amino-acids, the glucose being added as usual.

The growth obtained with glucose (0·5 %) and ammonium sulphate (0·3 %) present was again used as standard.

Table V.

Amino-acid (0·5 % solution)	Growth	
	Column 1 Amino-acid as source of carbon	Column 2 Amino-acid as source of nitrogen
Tryptophan ...	−	−
Tyrosine	−	−
Alanine	+ +	Trace
Phenylalanine ...	−	„
Histidine	−	„
Cystine*	+	„
Glycine	+ +	„
Asparagine ...	+ +	„
Glutaminic acid ...	−	„
Tyrosine + alanine	+ +	„
Tyrosine + histidine	−	−
Alanine + histidine	+ +	+
Histidine + glycine	+ +	Trace

* The low solubility of cystine enabled only a 0·1 % solution to be used. The flasks were tested at the end of the experiment and cystine found to be still available.

In column 1, *i.e.* in the absence of glucose, growth was obtained with alanine, glycine, asparagine, and to a less degree with cystine.

In column 2, *i.e.* in the absence of ammonium salts, the growth was erratic and was certainly never of the same order as with a purely inorganic source of nitrogen.

Discussion.

The experiments recorded in this paper show that it is comparatively easy to obtain growth of the organisms studied on a synthetic medium, but whether the metabolism and rate of multiplication of the organisms are normal under these conditions remains to be proved. In all cases it was found possible to obtain a marked improvement of the rate of growth by adding minute quantities of broth or of any other bios-containing substance. Experiments on the effect of bacterial accessory factors will be discussed in a later paper.

The absolute qualitative requirements of the organisms studied seem to be fairly simple. It is indeed true that the formula worked out for the inorganic requirements of the culture medium differs only slightly from that of Stephenson and Whetham [1924], but what the author wishes to emphasise is the necessity for keeping within narrow limits the range of concentration of any one ingredient. The presence of Cl ions is essential and a trace of $Ca(NO_3)_2$ seems to encourage growth.

It is impossible to exaggerate the importance of the progressive purification of the glucose. The filtrate after a first recrystallisation can be shown to have a distinct stimulating effect upon the growth: the filtrate after the fourth recrystallisation has none. A similar action was pointed out by Funk and Dubin [1921] in their experiments on the stimulating effect of "vitamin B" on the growth of yeast and bacteria. They state that treatment of the sugar with an absorbent eliminates the effect. This has recently been confirmed, and it was found that after treating the glucose with charcoal only two recrystallisations were necessary.

The addition of amino-acids to the synthetic glucose-inorganic salt medium, being an addition of heavier and more complex molecules, naturally complicates the problem. In this connection the work of Aubel [1920] is interesting but his view has not been confirmed. He studied cultures in which asparagine was the only source of carbon and of nitrogen, and compared them with cultures containing also glycerol, glucose and fructose. More ammonia was used and more growth was obtained by these additions. Glycerol gave a ninefold increase; this he believed to be due to the formation of pyruvic acid, which, given directly, had nearly twice the augmenting power of glycerol. His conclusion, therefore, was that carbon as —CO is most effective, then comes —CHO and lastly $—CH_2OH$. On the other hand, the conclusion to be drawn from the experimental section of this paper is that pyruvate is not more efficient in promoting growth than glucose, and is obviously inferior to glycerol.

The possibility that the controlling factor in the variation of growth is a

purely physical one, such as osmotic pressure, surface tension, etc., must be considered. That the lowering of surface tension within certain limits by an addition of fatty compounds may alter the type, but not the amount, of growth is shown in the following paper [Reader, 1927].

SUMMARY.

1. Experiments have been carried out to find the concentration of salts in a synthetic medium necessary to give maximum growth of certain micro-organisms. The organisms used were *Streptothrix corallinus*, a white *Streptothrix*, and *Sarcina aurantiaca* and a definite formula was decided upon.

2. Traces of manganese or iron did not increase the rate or amount of growth.

3. The following substances may replace glucose as source of carbon without loss of efficiency in the medium: glycerol, mannitol, arabinose, lactate, citrate, pyruvate and glycogen.

4. Amino-acids, as such, do not supply an improved source of nitrogen or carbon to the medium. Glycine, asparagine, alanine, and possibly cystine may supply carbon, but ammonium salts are a more efficient source of nitrogen.

I take this opportunity of expressing my thanks to Prof. J. C. Drummond for his helpful advice and criticism throughout this work, and to Mrs P. T. Kerridge for help with the determinations of hydrogen ion concentrations.

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CXXIV. THE RELATION OF THE GROWTH OF CERTAIN MICRO-ORGANISMS TO THE COMPOSITION OF THE MEDIUM.

II. THE EFFECT OF CHANGES OF SURFACE TENSION ON GROWTH.

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During the growth of cultures of *Streptothrix corallinus* upon the basal medium [Reader, 1927] with addition of broth or antineuritic concentrates (torulin), it was noticed that the organisms were distributed sometimes at the surface and sometimes throughout the volume of the fluid. This rather haphazard phenomenon suggested the effect of a surface tension factor, and experiments were accordingly devised to determine whether surface tension had an effect upon (1) the amount of growth, and (2) distribution or type of growth.

Previous work upon the subject of surface tension has been summarised by Frobisher [1926]. He draws attention to the significance of surface tension, not only in modifying the growth and morphology of bacteria, but perhaps also in influencing their cell-division, motility, and retention of Gram's stain. He found that differences in ability to grow at low surface tensions provided a simple method for distinguishing species of bacteria. Thus, by comparing the growth of organisms at low surface tensions, he succeeded in distinguishing *B. aerogenes* from *B. typhosus* and *B. morgani*; *B. diphtheriae* from *B. xerosis* or *B. hofmanni*, certain resistant types of streptococci from the more sensitive types. The raising (by the addition of charcoal) of the surface tension of Huntoon's hormone medium for pneumococci was, he claimed, responsible for an improvement of its growth-promoting properties.

Before the publication of Frobisher's results the only direct studies of the effect of surface tension on bacterial growth had been those of Larson and his colleagues. Larson, Cantwell and Hartzell [1919] claimed that the growth of pneumococci and streptococci was, in general, inhibited by surface tensions below about 50 dynes. They had also observed that the growth of *B. anthracis* was checked by a surface tension below 46 dynes, and that the sporulation of *B. subtilis* was similarly affected. It is, however, unfortunate that they did not substantiate their report by a detailed record of experiments. Larson and Larson [1922] studied the rôle of surface tension in pellicle formation. Larson and

Montauk [1923], cultivating tubercle bacilli in a medium containing soap and glycerol, noticed that the organisms grew indeed in this medium, producing a diffuse cloudiness, but appeared to have lost all pathogenicity, so much so that their injection into guinea-pigs was followed by nothing more than local temporary nodules. Tubercle bacilli in sputum seemed likewise to lose pathogenicity when treated with sodium ricinoleate. These results lend plausibility to the suggestion that low surface tension may have been a determining factor in the experiments of Calmette and his colleagues [1921].

Thus previous work shows that there is some evidence of a correlation between the amount of growth and the surface tension of the medium, but there appears to be no reference to any relation to the distribution of the organism throughout the medium.

Experimental.

Surface tension was determined by a drop volume method using a glass tip of approximately 3·8 mm. external diameter. The technique for cultivation and estimation of the growth of the organism, *Streptothrix corallinus* [Reader, 1926, 1, 2], was the same as described in the previous paper [Reader, 1927]. The broth employed was made from fresh ox-heart, trypsinised according to the method of Douglas. The antineuritic concentrates used were provided by Kinnersley and Peters [1925]. Small conical flasks (60 cc.) were used throughout, and the final volume of the medium was always made up to exactly 20 cc. in an attempt to keep the surface area constant.

Two types of measurement of surface tension were employed: (*a*) the surface tension between the medium and air, and (*b*) the interfacial tension between the medium and benzene. The latter measurement was undertaken under the belief that this figure might represent more nearly the change at the medium-cell interface. In view of the experiments of Hartridge and Peters [1922] the p_H was kept constant throughout. From Table I it will be seen that the percentage change is much greater in the interfacial surface than in the air surface for any one set of dilutions of medium. In all later experiments it was decided to use the interfacial medium/benzene figure, which seemed to be a more significant value and as it gave larger differences therefore decreased the percentage experimental error.

Table I.

	Pure medium	+1/200 broth	+1/20 broth
Volume of drop into air (cc.)	0·057	0·056	0·049
Volume of drop into benzene (cc.)	0·202	0·163	0·116

The figures in Table II are measurements of interfacial tension immediately *before* inoculating the flasks with the organism and incubating for 2 days at 31°. It was found that the interfacial tension against benzene did not change even during a prolonged growth period of 4 weeks, suggesting that the factor

lowering interfacial tension is not utilised in growth. α, β and γ represent in descending order the amount of growth in 48 hours gauged by opacity, standard flasks of a definite extract in varying concentrations being used for comparison. In these, growth was arrested after 48 hours by addition of formalin.

Table II.

Temperature = 16·8°
p_H = 7·4
Time of 1 drop = 40 seconds

Dilution of broth in salt-sugar medium	Volume of drop into benzene cc.	Growth in 48 hours at 31°	
1 in 0	0·057	α	Film
1 „ 1	0·058	α	„
1 „ 10	0·097	α	„
1 „ 20	0·116	α	„
1 „ 40	0·127	α	„
1 „ 60	0·134	β	„
1 „ 80	0·139	β	„
1 „ 100	0·144	γ	„
1 „ 200	0·163		„
1 „ 300	0·175		„
1 „ 400	0·186	decreasing	„
1 „ 500	0·199		„
1 „ 1000	0·202		Deposit
0 „ 1	0·202	↓	„

From Table II it may be observed that a gradual increase in the percentage of broth added to the synthetic medium increased growth up to a certain definite strength (1 in 40) when it was already equal to that obtained in a full broth culture; but whereas the addition of broth lowers the surface tension even when the dilution has reached only one part in 500, this is a figure widely divergent from that for maximum growth (1 in 40) recorded above. Further, when the interfacial tension is lowered even 1·5 % below its initial value (0·199 cc.) the character of growth changes from deposit to film.

Consequently it seemed probable that the amount of growth and the type of growth were not dependent upon the same factor. The film-growth seemed to be due to the lower surface tension of the medium rather than to the desire of the organism for more atmospheric oxygen, nitrogen, or other substance as is so often suggested.

Fig. 1 shows the type of curve obtained with descending dilutions of broth in a synthetic medium. Since the density of the solutions is not appreciably altered until a high concentration is reached, the surface tension can be considered for this experiment as directly proportional to the volume of drop except possibly for a slight error in the first point of the curve.

That the amount and the type of growth are in no way correlative is more definitely shown in Table III. The results there cited are typical examples of a series of fifty such experiments. The letters *A*, *B*, *C*, etc. represent various torulin concentrates and were used merely as sources of bacterial accessory factors. The figures in the second column are relative values only, 0·202 cc. being the value for pure inorganic-glucose medium. It will be noticed that

whenever the surface tension is low a surface film-growth is obtained; but the actual records of growth (α, β, γ, etc.) by no means correspond with the surface tension, or with the presence of films.

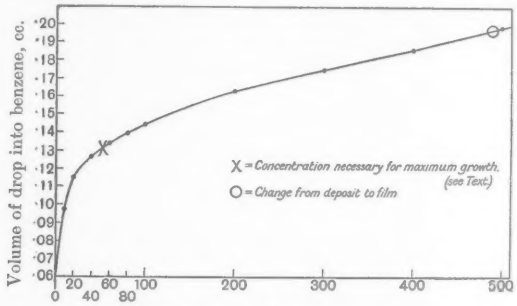

Fig. 1.

Table III.

Medium	Volume of drop (cc.)	Growth	Type of growth
A	0·105	α	Dense surface film
B	0·202	α	Rich deposit
C	0·151	$\alpha-$	Dense surface film
D	0·202	$\alpha-$	Rich deposit
E	0·127	β	Film
F	0·202	β	Deposit
G	0·184	γ	Film
H	0·199	γ	Deposit

Discussion.

The above experiments show that, when ordinary media are used, the surface tension of the fluid (within the limits recorded) does not affect the amount of growth. These conclusions may seem to be at variance with the work of Frobisher, of Larson, Cantwell and Hartzell [1919], and of Wolf [1923], but it must be remembered that their experiments involved the addition of substances such as soap and alcohol, and are, therefore, beyond the scope of this paper.

As there is no correlation between change in surface tension and amount of growth, it must be concluded that the growth-promoting effect of the medium used was not due to any surface action.

Summary and conclusions.

1. As the amount of growth of *Streptothrix corallinus* in synthetic medium containing additions of broth or antineuritic concentrates (torulin) is not related to the change of surface tension in air or interfacial tension against

benzene, the growth-promoting activity is not due to lowering of tension of the medium.

2. The type of growth (film or deposit) is correlated with change in interfacial tension of the medium against benzene.

I am indebted to Mr Kinnersley and Professor Peters for the torulin concentrates used throughout this work. I also wish to thank Professor Peters for his interest and helpful criticism.

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CXXV. EXPERIMENTS ON NUTRITION. VI.

BALANCE OF FOOD BY VITAMIN B.

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(*Received June 14th, 1927.*)

Our previous experiments [1922, 1923] on the rearing of chicks have indicated that the quantity of vitamin B required by the birds must bear a relation to the quantity of food consumed. These observations were thus similar to those described by Braddon and Cooper [1914]. Braddon [1907] had previously pointed out that in the epidemics of beriberi in the East the well-nourished succumbed first and were more liable to the disease than the underfed. Men with their greater energy output and consuming more rice than women were more subject to the disease. If men and women were kept upon the same fixed ration, as in institutions, both were equally susceptible. If they were supplied with equal quantities of other foodstuffs, an increase in the amount of white rice was followed by more cases of beriberi. The time of the appearance of the typical paralysis has been seen also to vary with the climatic conditions. Cold and wet weather, as well as exercise, hastened the onset, whilst warmth and rest delayed the appearance of the typical symptoms.

A connection between the actual quantity of food eaten and the time of onset of paralysis in birds receiving no vitamin B was first pointed out by Maurer [1907]. Cooper [1913] also observed that the disease appeared sooner in pigeons on larger amounts of white rice than in those on smaller amounts. Funk [1914] showed clearly that the onset of polyneuritis in pigeons occurred much sooner on 20 g. of rice than on 5 g., and also that on a constant amount of food polyneuritis appeared most quickly with starch or sugar, more slowly with caseinogen and lastly with fat.

The metabolic connection between carbohydrate and vitamin B was investigated in some detail by Braddon and Cooper [1914]. From their experiments with fowls it appeared that the greater the amount of carbohydrate, the greater was the amount of vitamin B required. Their work was never completed. No other direct experiments upon these lines had been made at the time that our experiments were started. The question of a quantitative

relation between food intake and vitamin B has seemed to us of so much importance in nutrition that our work during the past 3 or 4 years has been to test it, using each kind of foodstuff, protein, fat and carbohydrate.

In the meantime several other papers have appeared which show that vitamin B is concerned in the metabolism of food. Karr [1920] and Cowgill [1922] pointed out a relation between quantity of vitamin B and appetite. Osborne and Mendel [1922] found a connection between body weight and quantity of vitamin B. This connection has been elaborated by Cowgill [1922], Cowgill, Deuel and Smith [1925], Cowgill, Smith and Beard [1925] and by Beard [1925]. Randoin and Simonnet [1924] found a direct relation between carbohydrate consumption and amount of vitamin B. Hartwell [1924, 1925] and Reader and Drummond [1926] found a relation between quantity of protein in the food and amount of vitamin B. Our results point to the need of balancing all classes of foodstuffs by vitamin B, *i.e.* the supply of vitamin B must be in proportion to the calorie value of the food. Various pathological symptoms are seen which appear to originate from too little, or a shortage, of vitamin B in the food over a long period.

Experimental.

The rearing of chickens from day-old to maturity has been taken as the test to ascertain the balance of the three foodstuffs, protein, fat and carbohydrate by vitamin B. The rearing of ducks has been employed in additional experiments on balance of protein. In the case of balance of carbohydrate, pigeons and rats have also been used.

The housing of the chickens has been the same as described previously [1923]. Day-old chicks and ducks have been started in a foster mother and transferred, according to the weather, in 3 to 5 weeks, to a small house and later to a larger house, all the houses being provided with small runs. The houses have been on the roof of the building and exposed to the weather. Light Sussex chicks have been available for most of the experiments. Leghorns were used in one experiment and in another there was a variety of breeds. The pigeons were of the ordinary homer variety and were kept in pairs in cages, measuring approximately $4' \times 2' \times 2'$. The cages were kept side by side on the roof of the building and were fitted with a convenient perch. The rats have been kept in wire cages, measuring approximately $2' \times 1' \times 1'$, generally in pairs and housed in a warm room of the laboratory.

The birds and rats have always been fed *ad lib.* They have been provided with food in a tray, and as soon as it was finished, it was replenished. The food consumption could thus be recorded. All animals have been weighed weekly.

I. The balance of protein by vitamin B.

In our previous experiments [1923] it was found that chicks, groups VIII and IX, could be successfully reared on a diet composed of:

White rice	Marmite	Fishmeal	Cod-liver oil	
90	9	5	1	parts.

This diet presumably contained just enough vitamin B in the marmite to balance the rice and the small quantities of fishmeal and cod-liver oil. It has formed the basis of these experiments, which were devised on the following supposition.

Without any change in the above diet except raising the fishmeal to 30 parts, failure to rear chicks would indicate insufficient vitamin B, or some harmful effect of high protein in the food, and that the extra protein needed balancing. Then, by keeping the fishmeal constant at 30 parts and increasing the quantity of marmite, the capability of rearing chicks would show that the extra protein needed balancing with vitamin B and that the high protein was not in itself injurious.

First series of experiments.

Two groups of chicks, XII 5 and XII 30 were started together, the former as a control with 5, the latter with 30 parts of fishmeal. To the diet of group XII 5 was added the ash obtained by burning 25 parts of fishmeal, to represent the extra mineral matter supplied with the 30 parts of fishmeal to group XII 30.

Shortly afterwards, as a few more chicks became available, two more groups, XIII and XIII A, were started, both on low protein but with the proportions divisible into third quantities in order to be able to make up smaller amounts of food. These acted as a further control.

The rearing of group XII 5 was not so good as previously with groups VIII and IX. Groups XIII and XIII A reached maturity. The birds of group XII 30, except two, were not reared and these two were abnormal. More details are given below.

Further groups, XIV, XV, XVI, were then started in succession with increasing quantities of marmite. The results were worse with group XVI than with group XV. It was then suspected that the marmite was not so good as that previously used. Another control group, without and with ash from 25 parts of fishmeal, was then again tried. The results with these groups, XVII and XVII A, confirmed our suspicion that there had been some change in the marmite. Several other samples of yeast extract have in the course of these experiments been tested on pigeons and have been found to vary considerably (see later).

The series of experiments and the general results are best seen and compared by reference to Table I.

Table I.

Group	White rice	Marmite	Cod-liver oil	Fishmeal	No. of birds	Losses
XII 5	90	9	1	5	8	4
XII 30	90	9	1	30	8	6
XIII	90	9	1·5	6	3	0
XIII A	90	9·9	1·5	9	2	1
XIV	90	12	1	30	10	4
XV	90	14	1	30	12	1
XVI	90	16	1	30	10	6
XVII	90	10	1	5	9	5
XVII A	90	10	1	5	11	5

Composition of diets %.

Group	Water	Salt	Other ash	Fibre	Protein	Fat	Carbo-hydrate	Marmite	Calories per 100 g.	Ratio $\frac{\text{marmite}}{\text{calories}}$
XII 5	12·9	1·4	6·1	0·3	11·2	1·5	70·3	8·6	348	1 : 40
XII 30	13·2	1·7	5·7	0·2	20·8	1·8	56·9	6·9	335	1 : 49
XIII	12·9	1·4	2·7	0·3	11·6	2·0	69·3	8·4	350	1 : 42
XIII A	12·9	1·5	6·1	0·3	13·1	2·0	66·9	8·9	347	1 : 39
XIV	13·5	2·0	5·9	0·2	21·0	1·8	55·7	9·0	331	1 : 37
XV	13·7	2·1	6·0	0·2	21·3	1·8	55·0	10·4	330	1 : 32
XVI	13·9	2·3	6·1	0·2	21·4	1·8	54·4	11·7	328	1 : 28
XVII	12·9	1·5	2·6	0·3	11·5	1·5	69·7	9·4	347	1 : 37
XVII A	12·4	2·0	5·9	0·3	11·1	1·5	67·2	9·1	335	1 : 37

Although more birds were raised on the higher amounts of marmite in the food, the result in respect of balance of protein by vitamin B was not satisfactory and conclusive. The chickens which reached maturity were not normal in any of the high protein groups. They had bow legs, or were knock-kneed, and in some cases there were signs of leg weakness.

The most interesting and important details concerning these groups of birds were observed on *post mortem* examination. The details of the losses and *post mortem* examinations are given below.

Group XII 5. This experiment lasted for 27 weeks, from 15. viii. 1923 to 20. ii. 1924. The four losses occurred in the 4th, 5th, 7th and 18th weeks. A perforated gut was found in the first. In the second, the abdomen was full of liquid, the heart was enlarged, the gizzard small and gut empty. The third bird, before death, showed neck twist, which got better and then worse. It was chloroformed in the 27th week. Its caecum was very swollen. The fourth bird was pale and had drooping wings before death. The intestine was full of dark food material, the gizzard had thin walls and the kidneys seemed large. An extra bird introduced into this group at the age of 6 weeks showed leg weakness and bent legs in the 22nd week. It was chloroformed; its gizzard was thin-walled, intestines leathery and appendices swollen.

Slight leg weakness was noticeable in the 19th week and the group was kept longer, but distinct signs were not noticed in all the birds.

Group XII 30. This experiment was for 21 weeks, from 15. viii. 1923 to 9. i. 1924. The first loss in the 2nd week showed much liquid in the body cavity. The gut seemed normal. The second in the 4th week had an empty intestine: the bones seemed soft. The third in the 5th week had distinct leg weakness. The leg joints were large. The gut was almost empty. The fourth in

the 15th week was very wasted. The knee joints were swollen and legs twisted. There was very little muscle on the gizzard. The fifth and sixth died in the 20th week. There were hard lumps in the caeca with stoppage. The gizzards had thin walls. The two surviving birds were kept to the 26th week. No gut symptoms were noticed at autopsy. The ovaries were small. The birds were abnormal in appearance.

Group XIII. The experiment was for 25 weeks, from 11. ix. 1923 to 20. ii. 1924. One bird showed neck twist in the 11th week, but got better. There seemed signs of leg weakness in the 16th week, but it did not get worse. This group with the two remaining hens from group XII 5 were kept altogether for 12 months without any symptoms. The marmite was raised to 10 parts for the latter period.

Group XIII A consisted of only two chicks, one of which was very small and feeble at the start and should not be included. The surviving chick at the 8th week was put in group XIII.

Group XIV. This experiment was continued only for 10 weeks, from 6. ii. 1924 to 16. iv. 1924. Two birds showed badly twisted legs in the 4th week and were chloroformed. They had pale combs and swollen joints. The caeca contained masses of food which caused stoppage. Two further birds showed crooked legs in the 5th and 6th weeks and were chloroformed. The second had water in the body cavity; its intestines and appendices were swollen. All the birds at the 8th week were very weak on their legs. The marmite was raised to 16 parts, but they did not get better and were chloroformed at the 10th week.

Group XV. This experiment lasted for 9 weeks, from 26. iii. 1924 to 26. v. 1924. The loss in this group was in the 2nd week. The bird had a crooked leg and was chloroformed. It had an unabsorbed yolk sac. The other birds had bow legs, or were knock-kneed and weak on their legs. On this account the experiment was not continued longer.

Group XVI. These were Leghorns. The experiment was for 7 weeks, from 22. iv. 1924 to 18. vi. 1924. This was an unsatisfactory group as seven birds died in the 1st week from unabsorbed yolk sacs, which pointed to weakness in the other birds. One bird developed a crooked leg in the 5th week and was chloroformed. Another died in the 7th week. Its abdomen was full of water, but the intestines seemed normal; its heart was normal. All the birds were very ill and the experiment was discontinued.

Group XVII. This experiment lasted for 20 weeks, from 22. iv. 1924 to 10. ix. 1924. The first two losses occurred in the 3rd week. Both appeared to have stoppage of the gut. The third loss was in the 4th week. Both appendices of the bird were clogged with rice. At the 6th week the birds remaining from group XVII A were put in with this group. Two deaths occurred in the 19th week; no special symptoms were noticed. Another loss was in the 21st week. This bird had an enlarged heart with clot in the pericardium; the connective tissue in the abdomen was leathery.

Group XVII A was fed for 6 weeks, from 22. iv. 1924 to 28. v. 1924. The first loss was in the 2nd week. The liver was pale and the gall bladder large. The second and third losses were in the 3rd week. One showed a stoppage of the intestine and the other a distended gut with pale liver and gall bladder. The fourth and fifth died in the 4th week. In one the yolk sac was not completely absorbed and there was stoppage near this point. In the other, the gut was full of hard faecal matter, the liver and gall bladder were pale. The surviving birds were now put in with group XVII.

The general results of these experiments were not conclusive. On the whole the birds seemed to thrive better with the extra marmite in the food. The birds with the high protein diet were always abnormal. It was difficult to account for the abnormalities. Several possibilities could be suggested. They may have been due to the high salt content of the diet, derived from the marmite and from the fishmeal, or to the high amount of the protein, or possibly to too little cod-liver oil, or to a combination of several conditions. The extra mineral matter introduced with the higher amounts of fishmeal was of no value in preventing leg weakness and this does not support the claim that lack of mineral matter is the cause of deficiency diseases. In former experiments a diet had been fed to chicks with an equally high protein content, and no ill-results were observed. It was therefore difficult to attribute the errors to high protein. The amount of cod-liver oil though about 0·7 % of the diet would also appear adequate.

Experiments with ducks.

Some trials of a similar kind were made about the same time with groups of ducklings. As it was previously found that ducks required more marmite than chicks, the quantity of marmite was placed at a higher figure at the start. The diets and general results are given in Table II.

Table II.

Group	White rice	Marmite	Cod-liver oil	Fishmeal	No. of birds	Losses
D, III	90	12	1	30	12	4
D, IV	90	16	1	30	4	0
D, V	90	20	1	30	11	6
D, VI	90	40	1	30	7	0

Ducks III were not well and were shaky on their legs in the 2nd week. On this account, the marmite was slowly raised (every 2 weeks) to 20 parts. It seemed that it was only by these means that the ducks could be reared.

The first loss showed a pale fatty heart; the caecum had stoppage and the appendices were blown. There was stoppage of the gut with the cloaca distended with gas in the second loss. No cause could be found for the third death. The gut was normal in the fourth except that one appendix was full. The interior was pale.

Ducks IV were very shaky on their legs and were combined with ducks V at the 10th week.

Ducks V. All but one of the losses in this group occurred in the 3rd week. The only symptoms seemed to be swollen and pale kidneys. There was a loss in the 10th week apparently from tuberculosis.

As a final trial ducks VI were fed with a very high amount of marmite. They were all reared to the 10th week and were quite normal.

Altogether there was more evidence for balance of protein by vitamin B in these experiments than in the corresponding ones with chickens.

Second series of experiments with chickens.

Owing to the apparent deterioration of the marmite, this series of experiments was made with dried yeast as source of vitamin B. Its use would also get rid of the possibility of the high salt content of the previous diets. It had been found previously [1923] that dried yeast contained about half the amount of vitamin B present in the former marmite, so that 20 parts were taken as the equivalent of 10 parts of marmite as the minimum quantity to balance the basal diet. This series was carried out in the reverse order starting with high yeast and diminishing as shown in Table III.

Table III.

Group	White rice	Dried yeast	Cod-liver oil	Fishmeal	No. of birds	Losses
XIX	90	32	1	30	11	0
XX	90	28	1	30	11	0
XXI	90	24	1	30	10	0
XXII	90	20	1	30	10	2

Composition of diets %.

Group	Water	Ash	Fibre	Protein	Fat	Carbo-hydrate	Calories per 100 g.	Yeast	Ratio $\frac{\text{yeast}}{\text{calories}}$
XIX	10·1	5·8	0·2	24·8	1·7	56·8	350	21	1 : 17
XX	10·3	5·7	0·2	24·3	1·7	57·1	349	19	1 : 18
XXI	10·5	5·6	0·2	23·7	1·8	57·5	350	17	1 : 21
XXII	10·8	5·6	0·2	23·2	1·7	57·9	348	14	1 : 25

All these experiments were made in succession for a duration of 10 weeks during the time 27. vi. 1924 to 16. ix. 1924.

Though no losses occurred in the first three groups, all the birds at the age of 10 weeks had either bow legs or were knock-kneed, but otherwise they were fairly normal.

In group XXII, in one of the birds lost, there was a twist in the intestine, probably an abnormality at the start as the bird died in the 2nd week. In the other bird the gut seemed normal, but the kidneys were large and uric acid was found by testing in the ureters and cloaca.

The results again were not clear. The high protein seemed to have a deleterious effect. The birds seemed to be distinctly better on the higher amounts of dried yeast.

Third series of experiments.

This series was made to ascertain the effect of increasing quantities of protein in the diet so as to find out whether the high protein was producing the bow legs and other symptoms. The amount of dried yeast was kept constant (Table IV).

Table IV.

Group	White rice	Dried yeast	Cod-liver oil	Fishmeal	No. of birds	Losses
XXIII	90	20	1	5	9	0
XXV	90	20	1	10	9	2
XXVI	90	20	1	15	7	0
XXVII	90	20	1	20	9	4
XXVIII	90	20	1	25	13	2
XXIX	90	20	1	30	8	2
XXX	110	20	1	5	12	4

Composition of diets %.

Group	Water	Ash	Fibre	Protein	Fat	Carbo-hydrate	Calories per 100 g.	Yeast	Ratio yeast / calories
XXIII	9·9	2·8	0·2	15·1	1·5	66·9	350	17·2	1 : 20
XXV	10·1	3·4	0·2	17·0	1·5	64·1	346	16·5	1 : 21
XXVI	10·3	4·0	0·2	18·7	1·6	61·6	344	15·9	1 : 22
XXVII	10·5	4·6	0·2	20·3	1·7	59·2	342	15·3	1 : 22
XXVIII	10·6	5·1	0·2	21·8	1·7	57·1	339	14·7	1 : 23
XXIX	10·8	5·6	0·2	23·2	1·8	55·1	338	14·2	1 : 24
XXX	10·1	2·4	0·3	13·9	1·3	69·0	352	14·7	1 : 24

The last group acted as a control with extra white rice equal to 30 parts of fishmeal. All the groups were kept for 20 to 23 weeks, from 21. vii. 1924 to 4. v. 1925.

Group XXIII. All the birds were reared without any signs of illness. They were actually continued for another 10 weeks with one cock bird and laid eggs. They had a tendency to eat the eggs. On this account the cod-liver oil was raised to 2 parts for a further 7 weeks and then to 3 parts for a period of 5 weeks. This increase lessened the desire to eat eggs.

Group XXV. The *post mortem* examination of the two birds which died in this group did not reveal any particular symptoms. Almost the same diet was used again in group XXXIII and the birds were reared satisfactorily.

Group XXVI. This group was reared quite satisfactorily.

Group XXVII. This group turned out to be composed only of cock birds. The first loss was in the 4th week. The gut contained compact food masses and the appendices were swollen. The other three birds had crooked legs at the end of the period of experiment and were chloroformed. All three were found to have swollen appendices with dark food residues in them; the livers were large and fatty. The other birds seemed normal.

Group XXVIII. This group looked ill in the 7th week and the birds were shaky on their legs. Several showed only small increases in weight at the 13th week. The first loss was in the 19th week. The bird had enlarged kidneys, stopped gut and very swollen appendices. The other died in the 23rd week

and had also stopped gut and swollen appendices. The hen birds of this group showed bow legs or legs with slight twist.

Group XXIX. Like group XXVIII the birds showed signs of illness in the 7th week. All the birds were shaky on their legs and the legs of many were crooked. The first loss was in the 19th week; examination showed a flabby gizzard and one swollen appendix. The other bird died in the 20th week. Its appendices were swollen, the liver was fatty and there was much fat upon the heart.

Group XXX. This group, a control group to XXIX, showed its first loss in the 4th week. There was some stoppage of the large intestine and the appendices were full of black food residues. The second loss was in the 5th week. There was undigested rice in the large intestine and the appendices were large. The third bird died in the 6th week. The large intestine was full of food residues, but the appendices were empty. The fourth bird showed bent legs in the 9th week; it did not get better and was killed in the 17th week. Its abdomen was found full of water and gelatinous matter.

All birds of the group were more or less shaky on their legs at the end of the experiment.

It appears from these experiments that high protein affects the birds and may be the cause of the bow legs or knock knees. These symptoms do not appear until the fishmeal reaches 20 parts, or about 20 % of the food. A clear demonstration of the need of balancing the protein by vitamin B does not seem possible without producing ill-effects from too much protein. Excluding the leg abnormalities, the *post mortem* findings were very similar with the smaller amounts of vitamin B, and were generally concerned with the intestines and appendices and sometimes the heart.

Fourth series of experiments.

During the course of the previous experiments others were made with small amounts of cod-liver oil and it was being noticed that 0·4 to 0·6 % was too little and later that 0·5 to 0·7 % of cod-liver oil was enough to rear birds without any signs of illness. In the experiments with low fishmeal, the amount of cod-liver oil was 0·9 %. This amount became reduced to 0·8 and 0·7 % as the fishmeal increased in quantity. It was possible that too little cod-liver oil was the cause of the leg abnormalities. This series was tried with 2 % of cod-liver oil so as to avoid ill-effects from too little. The diets used in these groups were made to contain 2 % of cod-liver oil and the whole diet was made out to a total of 100 parts. The diets are shown in Table V.

Table V.

Group	White rice	Dried yeast	Cod-liver oil	Fishmeal	No. of birds	Losses
XXXIII	74	16	2	8	10	1
XXXV	78	12	2	8	10	2
XXXIV	56	12	2	30	10	2
XXXVII	52	16	2	30	12	3

Composition of the diets %.

Group	Water	Ash	Fibre	Protein	Fat	Carbo-hydrate	Calories per 100 g.	Ratio yeast / calories
XXXIII	10·0	3·3	0·2	16·6	2·7	66·9	368	1 : 23
XXXV	10·4	3·0	0·2	15·1	2·7	68·4	367	1 : 31
XXXIV	11·1	6·8	0·2	27·0	3·3	50·6	349	1 : 29
XXXVII	10·7	7·3	0·2	28·5	3·3	49·1	349	1 : 22

The diet of group XXXIII was the same as that of group XXV except for the cod-liver oil. With group XXXV this group acted as control to the two groups with high protein.

Group XXXIII. The one loss in this group could be accounted for by an accident. All the birds were perfectly fit and this one showed one morning peculiar symptoms of turning somersaults. The *post mortem* examination showed normality in all the organs. The bird was probably pecked by another or had some injury to its head.

Group XXXV. The losses were in the 5th and 6th weeks. The first was shaky on its legs and showed slight leg weakness. At autopsy, decomposing food was found in the large intestine extending into the small intestine. The appendices were full and swollen. The second bird had excessive amounts of fat on the abdominal organs. The intestines were blocked and the heart was very large. The other birds of the group were quite normal.

Group XXXIV. All the birds in the group showed leg weakness and looked very ill. The experiment was consequently stopped in the 15th week.

The two losses were in the 5th and 7th weeks. Both birds were chloroformed on account of their leg weakness. The first had decomposing food in the large intestine and swollen appendices; the gizzard was flabby and full of water. There was gas in the appendices of the second bird.

Group XXXVII. The experiment was stopped at the 16th week as all the birds showed bow legs. The first loss was in the 3rd week. The intestines and appendices were full of dark food residues. The second died in the 13th week. There was ballooning of the gut near the gizzard. In both birds the hearts seemed small.

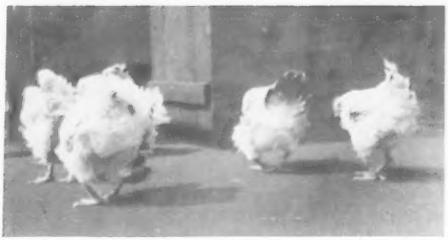
Group XXXVII. These birds were generally in better condition than those of group XXXIV. They were able to walk whereas those of group XXXIV could not do so. It would have been quite possible to continue group XXXVII, but no further information was expected.

As the cod-liver oil was raised in this series, the abnormalities in the legs thus appeared due to high protein in the diet. The birds were distinctly better with the higher quantity of yeast.

The definite proof of the need to balance the protein in the diet is not possible of attainment with chicks. The experiments, however, indicate in a general way that with high protein the birds thrive better with a higher quantity of vitamin B as supplied in dried yeast.

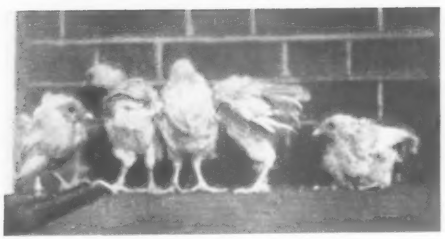
The photographs show the birds of groups XXXIII and XXXIV, the latter with the bow legs noticed with high protein in the diet (Plate VI, figs. 1 and 2).

1
Group XXXIII

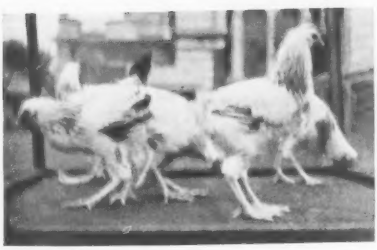


2
Group XXXIV

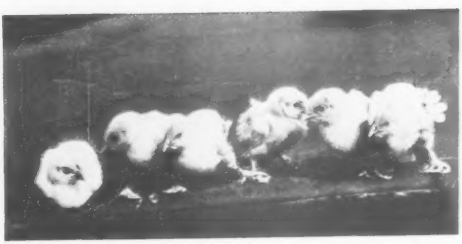
3
Group XXXVI



4
Group XLIV



5
Group XLVII A



6
Group M VI p. 943).

UNIV. OF MICH.

II. The balance of fat by vitamin B.

Our former observations that excess of cod-liver oil in the diet of chicks produced ill-effects and that these were counteracted by giving additional marmite were the starting-point of these experiments. Excess of cod-liver oil in the diet of chickens has also been found in agricultural experiments to be detrimental to their health and an endeavour was made to prove that vitamins were therefore of little value in nutrition. There was supposed to be an ample supply in the various foods given in agricultural practice.

In our experiments on high fat diets the use of a large quantity of cod-liver oil was avoided by working with cotton-seed oil. This oil does not contain any appreciable amount of vitamin A. Any ill-effect produced must therefore be attributed to the oil.

The diets for these experiments were planned on the same basis as those in the fourth series on protein balance. The experiments were carried out in succession as the results of each became clear. The series is shown in Table VI.

Table VI.

Group	White rice	Dried yeast	Cod-liver oil	Fishmeal	Cotton-seed oil	No. of birds	Losses
XXXVI	58	12	2	8	20	15	0
XXXVIII	54	16	2	8	20	10	1
XLI	50	20	2	8	20	10	1
XLIV	60	10	2	8	20	8	5

Composition of the diets %.

Group	Water	Ash	Fibre	Protein	Fat	Carbo-hydrate	Calories per 100 g.	Ratio $\frac{\text{yeast}}{\text{calories}}$
XXXVI	8·1	2·8	0·2	13·8	22·6	52·2	481	1 : 40
XXXVIII	7·7	3·2	0·2	15·3	22·6	50·8	481	1 : 30
XLI	7·4	3·5	0·2	16·8	22·6	49·3	481	1 : 24
XLIV	8·3	2·7	0·2	13·1	22·6	52·9	481	1 : 48

Group XXXVI. Though there were no losses in this group, the birds looked abnormal throughout the time of the experiment, which lasted for 26 weeks, from 26. v. 1925 to 19. x. 1925. They were greasy to the touch and generally dirty in appearance. Slight leg weakness was noticeable in the 3rd week. It became more obvious in the 7th and 8th weeks and persisted until the end of the time. The birds were always sitting during the day. The chief feature of their appearance was the poor feathering; some birds were almost naked during the time of the trial.

Group XXXVIII. The birds of this group in contrast with those of the preceding one were always quite clean to look at and were not greasy to the touch. Their feathering was about as good as that of a normal bird. The one loss occurred in the 13th week. There was fluid in the body cavity and the intestines and appendices contained dark food material.

Group XLI. This group was tried to see if still more dried yeast would be of greater benefit in balancing the fat. No advantage was noticed. The loss in this group was in the 5th week and was due to pneumonia.

Group XLIV. The object of this experiment was to see the effect of less dried yeast than used in group XXXVI. The dirty and greasy appearance of the birds was again manifest. The first loss was in the 8th week. The heart was enlarged and covered with gelatinous matter. The appendices were swollen; the kidneys and liver pale. All the birds were very shaky at the 16th week and quivered as they stood. The second loss was in the 17th week; before death the bird showed distinct leg weakness. It was very fat in the abdominal region. The heart was also covered with fat. The appendices contained black food residues, and there was stoppage at their junction with the intestine.

The group was kept longer than the other groups to see the effect of long continuation of the diet. In the 23rd week the third loss occurred. The flesh of the bird was very wet, and there was fluid in the abdomen. It seemed as if there had been a burst blood vessel as blood clots were also noticed. The fourth bird died in the 26th week. It was also dropsical, its interior being full of gelatinous matter. The heart, kidneys and liver seemed very large and there were black food residues in the intestine. The fifth died in the 27th week. The inside was again very wet. Heart and liver were much enlarged. There was decomposing matter in the intestines and the appendices were swollen.

The other birds at the conclusion of the experiment on examination were more healthy in appearance. They showed a large amount of fat in the abdominal region and the heart of one was distinctly larger than normal.

There was a distinct difference in the rate of growth of the birds in these groups. It corresponded with the amount of dried yeast in the food, being greatest in group XLI and least in group XLIV. This could be attributed to the slightly greater amount of protein supplied in the food, but is more likely due to appetite which has so often been shown to depend on the quantity of vitamin B. The rate of growth is shown in Table VII.

The series of experiments clearly points to the need of balancing the fat in the food by vitamin B. The photographs of groups XXXVI and XLIV show the condition of the birds. The poor feathering of group XXXVI is not so clear in the picture as it was during the course of the experiment (Plate VI, figs. 3 and 4).

III. The balance of carbohydrate by vitamin B.

The experiment with group XXX in which extra white rice was included in the food in contrast with group XXIII had already shown that the whole of the carbohydrate needed balancing by dried yeast. In order to show the need of balancing more distinctly a series was started using the groups XXXIII and XXXV as the controls and feeding with diminishing quantities of dried yeast, until the amount was found which was not sufficient to rear the birds satisfactorily. The series is shown in Table VIII.

As stated above, group XXXIII was a perfectly normal group, and the losses in group XXXV have been described. It was not expected at the start that

Table VII. *Average weekly weights (g.) of birds in the balance of fat experiments.*

	Cocks				Hens			
Group	XXXVI	XXXVIII	XLI	XLIV	XXXVI	XXXVIII	XLI	LXIV
No. of birds	11	6	5	6	4	3	4	2
Week								
0	35	39	40	34	38	36	39	33
1	50	65	65	51	52	60	58	52
2	73	105	93	70	78	95	80	72
3	103	142	151	94	111	132	121	110
4	139	177	212	135	142	168	165	156
5	189	214	277	179	189	215	195	193
6	251	258	363	261	240	275	259	252
7	322	339	428	317	304	363	314	302
8	390	449	476	410	357	463	349	360
9	460	551	625	522	422	523	462	410
10	548	647	673	613	509	598	536	475
11	692	732	785	717	594	656	605	572
12	793	892	918	779	645	806	735	600
13	895	1058	1029	918	695	898	839	705
14	1030	1168	1260	999	811	972	999	762
15	1146	1310	1302	1092	900	1065	1054	785
16	1359	1515	1505	1208	1026	1233	1160	872
17	1462	1682	1723	1391	1114	1390	1317	715
18	1668	1852	1801	1581	1237	1497	1375	840
19	1880	1959	1986	1645	1341	1540	1477	850
20	2055	2111	2134	1802	1437	1605	1597	1000
21	2068	2215	2380	1870	1505	1735	1711	1030
22	—	—	2538	1993	—	—	1825	1100
23	—	—	2697	2136	—	—	1961	1200
24	—	—	—	2170	—	—	—	1245
25	—	—	—	2242	—	—	—	1360
26	—	—	—	2442	—	—	—	1435
27	—	—	—	2445	—	—	—	1585
28	—	—	—	2522	—	—	—	1655

Table VIII.

Group	White rice	Dried yeast	Cod-liver oil	Fishmeal	No. of birds	Losses
XXXIII	74	16	2	8	10	0
XXXV	78	12	2	8	10	2
XL	80	10	2	8	11	1
XLII	82	8	2	8	11	1
XLV	86	6	2	8	10	1
XLVI	86	4	2	8	13	0
XLVII A	88	2	2	8	12	2
XLVII B	88	2	2	8	9	6
L	89	1	2	8	5	5
XLIX	77	6	2	15	10	1

Composition of the diets %.

Group	Water	Ash	Fibre	Protein	Fat	Carbo-hydrate	Calories per 100 g.	Ratio $\frac{\text{yeast}}{\text{calories}}$
XXXIII	10·0	3·3	0·2	16·6	2·7	66·9	368	1 : 23
XXXV	10·4	3·0	0·2	15·1	2·7	68·4	367	1 : 31
XL	10·6	2·8	0·2	14·4	2·8	69·1	368	1 : 37
XLII	10·7	2·7	0·3	13·7	2·7	69·8	368	1 : 46
XLV	10·9	2·5	0·3	12·9	2·7	70·5	367	1 : 61
XLVI	11·1	2·4	0·3	12·2	2·6	71·3	367	1 : 92
XLVII	11·3	2·2	0·3	11·4	2·6	72·0	366	1 : 183
L	11·4	2·1	0·3	11·1	2·6	72·4	366	1 : 366
XLIX	11·1	3·8	0·2	16·7	2·9	64·9	362	1 : 60

so many groups would be required before the balance would be shown by losses of birds.

Group XL. Twenty-one weeks, from 10. vii. 1925 to 5. xii. 1925. One bird in the 9th week showed distinct leg weakness. It was given 1 g. of marmite and was cured next day, which showed that the leg weakness was due to lack of vitamin B. It never showed the symptoms again and was quite well at the end. The bird which died had an abscess under the skin which caused peritonitis. The excess cocks were killed in the 22nd week. Examination showed them to be quite normal in their appearance. One cock and the hens were then kept altogether for 52 weeks. They laid eggs, which were mostly unfertile. One hen died in the 42nd week. There were masses of fat in the body cavity, every organ being covered with fat. The connective tissue was leathery and the heart pale.

The group was considered quite normal throughout the experiment.

Group XLII. Twenty-two weeks, from 10. viii. 1925 to 11. i. 1926. The loss in this group took place in the 5th week. The bird had leg weakness and died suddenly. All the birds in the 8th week showed poor feathering and one had a head twist. They scattered their food. At the 18th week the birds looked pale and seemed to have signs of leg weakness. The excess cocks were killed in the 23rd week. All were very fat in the abdominal region and the appendices were full of pasty black food residues. The connective tissue was leathery. One cock and the hens were kept on longer. The cock died suddenly in the 31st week. It had a pale liver and large heart. The hens were put in with those of group XLV. The eggs laid were mostly unfertile, and the fertile ones did not hatch.

The group could not be considered as a perfectly normal one.

Group XLV. Twenty-one weeks, from 11. xi. 1925 to 7. iv. 1926. There was one loss in the 5th week. Hard lumps of food were found in the intestine and the heart was pale. The birds generally did not look as well as those observed in the previous groups and also scattered their food. The excess cocks were killed in the 22nd week. In all these birds the gizzards were flabby, but only small amounts of fat were noticed around the gizzards and hearts. The hens and one cock were kept on to the 38th week. Most of the eggs laid were unfertile; the fertile ones gave a few hatches but the chicks soon died, being in very poor condition.

The group was not a perfectly normal one.

Group XLVI. Twenty-one weeks, from 5. v. 1926 to 29. ix. 1926. Though there were no losses in this group, the birds that reached maturity were not normal. From the 3rd week the birds always left the rice of their food, but were not given fresh food until it was eaten. One bird in the 8th week had leg weakness, but got better. At the 20th week the birds looked very ill. Five showed distinct drooping of the wings and three had weak legs and were distorted to one side. They were killed in the 21st week. At *post mortem* examination the chief feature of all the birds was the enormous amount of

fat around the necks and around all the internal organs. In many the hearts were enlarged. The intestines had pasty black food residues in them and the appendices were in some cases swollen.

The group was quite abnormal.

Group XLVII A. Twenty-one weeks, from 10. v. 1926 to 4. x. 1926. The first loss took place in the 14th week. The interior of the bird was very fatty and the appendices were slightly swollen. The heart, liver and kidneys were pale. The second loss was in the 17th week. The inside of the bird was very fat and the heart large. The birds in general did not feather well and were distinctly shaky on their legs. At *post mortem* examination the insides were found to be very fat, all the organs being more or less covered. In some the hearts were large and the intestines and appendices swollen. The birds were not by any means normal (see Plate VI, fig. 5).

Group XLVII B. Twenty-six weeks, from 29. x. 1926 to 29. iv. 1927. The survival of the birds of group XLVII A was so remarkable that a further experiment was made with the same diet. Six birds died during the course of 13 weeks. In each case the heart was large. In most the intestines had black food residues and the appendices in several were not normal. In these young birds there was not the visibly large amount of fat in the abdomen.

The three birds surviving to 26 weeks were hens. At *post mortem* examination they were found to be abnormally fat in the abdominal region. A mass of nearly 200 g. of fat could easily be removed by hand from one. They had large hearts and fluid was found in the pericardium of all three.

The losses and abnormality of the last three birds clearly show that 2 % of dried yeast is quite insufficient for balancing the food.

Group L. This group was started because the birds of group XLVII A lived so long. All birds except one died within 6 weeks. The single bird which survived lived for 20 weeks and then died suddenly. Its interior was not fat, but the small intestine was swollen and the heart large. The bird always ate very little and this may account for its long survival.

Group XLIX. Twenty-one weeks, from 6. viii. 1926 to 31. xii. 1926. This group was tried to see if more protein in the diet of the birds with 6 % of dried yeast, which were apparently normal in external appearance, would have any special effect. It was thought that high protein in the diet had a harmful effect. One bird died in the 3rd week from an unabsorbed yolk sac. The birds already in the 7th week looked ill and never seemed perfectly fit. They always panted after being caught for weighing. At *post mortem* examination some of the birds were found to be very fat. Several had crooked breast-bones. The appendices generally contained pasty food and black food residues were often observed in the intestines. In many the hearts were obviously large. The birds were not normal, though there were no distinctive abnormalities. High protein appears to have an influence upon the health and appearance.

These experiments show definitely that 2 and 4 % dried yeast are insufficient to balance the carbohydrate in the food. With 6 % dried yeast the

birds which reached maturity were not absolutely perfect. A really good result was only obtained with 10 % of dried yeast. The weights of the birds in the different groups as shown in Table IX also show quite definitely that 2 and 4 % are too little. The weights of the birds in the other groups, except possibly group XLII, are much the same each week. 6 % is the minimum of dried yeast and 10 % is a good quantity.

Table IX. *Average weekly weights of birds in the balance of carbohydrate experiments.*

Cocks

Group ...	XXXIII	XXXV	XL	XLII	XLV	XLVI	XLVII A
% of yeast	16	12	10	8	6	4	2
No. of birds	2	5	6	6	5	7	8
Week							
0	42	39	39	33	35	40	36
1	52	59	59	48	63	54	51
2	101	95	102	76	99	88	69
3	141	157	157	117	150	108	93
4	174	248	225	185	221	146	126
5	291	370	306	249	325	196	163
6	447	451	368	329	405	249	209
7	525	592	490	403	510	325	266
8	777	707	637	489	666	438	368
9	862	897	825	620	805	583	447
10	1122	1039	1002	743	1000	684	528
11	1317	1266	1156	963	1182	794	672
12	1522	1435	1343	1144	1390	1023	818
13	1730	1715	1533	1333	1479	1184	958
14	1955	1982	1758	1537	1695	1381	1103
15	2125	2140	1880	1779	1846	1584	1299
16	2335	2332	2207	1930	2139	1785	1508
17	2480	2589	2363	2119	2303	1979	1711
18	2655	2799	2584	2234	2498	2119	1889
19	2765	3074	2711	2412	2666	2240	2075
20	3080	3215	2955	2553	2684	2492	2248
21	3215	3225	3083	2778	2707	2359	2131

Hens

Group ...	XXXIII	XXXV	XL	XLII	XLV	XLVI	XLVII A
% of yeast	16	12	10	8	6	4	2
No. of birds	7	6	5	4	4	6	4
Week							
0	37	37	39	31	35	41	36
1	54	59	57	49	61	54	54
2	93	99	89	76	99	85	70
3	137	165	130	117	147	102	93
4	199	232	184	189	205	140	122
5	305	347	251	249	294	176	158
6	424	434	319	327	379	225	200
7	546	546	406	405	472	285	262
8	697	670	507	482	632	392	341
9	791	809	642	631	750	496	396
10	982	930	762	752	909	584	454
11	1141	1087	898	937	1061	695	554
12	1309	1202	1015	1076	1214	849	655
13	1441	1397	1154	1210	1300	977	754
14	1541	1532	1320	1387	1446	1133	848
15	1649	1607	1388	1541	1528	1287	952
16	1819	1678	1634	1624	1692	1444	1126
17	1964	1838	1720	1724	1771	1569	1290
18	2101	1944	1870	1844	1945	1681	1410
19	2164	2111	1963	1977	2109	1775	1531
20	2357	2217	2059	2045	2125	1932	1654
21	2457	2213	2120	2180	2420	1877	1660

Experiments with pigeons.

During the course of the various trials with chickens similar experiments upon balancing the carbohydrate of the food with vitamin B were made with pigeons. Their diet was composed of white rice, later white flour, fishmeal and marmite, or dried yeast. Cod-liver oil was omitted, as it had previously been found that pigeons did not need cod-liver oil in their food [1923].

First series.

Two pigeons were placed on 1. i. 1924 upon the same diet as group XII 5, *i.e.*

White rice	Marmite	Fishmeal
90	9	5

At the end of 12 weeks it was discovered that they were two hens. A cock was substituted for one of them and the pair kept on this diet for 10 weeks. During this time two lots of two eggs were laid by the hen. The first lot was unfertile, the second was fertile and squabs were hatched but soon died. In order to improve the hatching and rearing, the quantity of marmite in the food was raised to 10 parts for the next 8 weeks, and then to 12 parts for a further 7 weeks. The eggs that were laid during these periods were deserted by the birds, one lot later disappeared, possibly eaten by the birds. As probably some other food constituent was needed for reproduction, a further increase in the marmite was not tried.

Altogether one bird was kept for 23 weeks and the other for 10 weeks with 9 parts of marmite and the two birds at the end showed a slight increase in weight. This quantity could therefore be considered ample for maintenance.

In order to find out the least quantity of marmite that would suffice to maintain the birds, the quantity was reduced to 4 parts. At the end of 6 weeks both birds had lost over 100 g. in weight and looked very ill. They had lost their appetite and scattered their food.

The marmite was raised to 6 parts; the food was now eaten without scattering and both birds increased in weight. It seemed that 6 parts of marmite was the quantity to balance the rice.

Our former figure for marmite in the same diet was 3 parts. The marmite was thus not so rich in vitamin B as in the former year. This was already indicated in the experiments with chickens on the balance of protein.

Samples of other yeast extracts.

This pair of pigeons, as seen above, were able to discriminate by scattering of the food and loss of appetite with concomitant loss of weight whether the food contained enough yeast extract or not. They were used for a rough test of several other samples of yeast extract which we were able to investigate.

The first sample, designated by us as *A*, was started at the level of 3 %. The birds lost weight and did not like the food. On raising the proportion to 3·5 % the birds recovered their appetite and weight.

The second sample, designated by us as *B*, was started at 3·5 %. The birds scattered the food and declined in weight. The amount was first raised to 4 %, then to 4·5, then to 5 and to 6 %. At this level the birds ate the food without scattering, regained their appetite and increased in weight. On again reducing to 5 %, they showed loss of appetite and loss of weight.

The third sample, designated *C*, was started at 5 %. The birds scattered the food and lost weight; on raising to 8 parts they recovered. On reducing to 7 they lost weight again, but recovered on raising to 10 %. The hen now suddenly died and the experiment was stopped.

The fourth sample of yeast extract, designated by us as *V*, was tried on another pair of birds. On 3 % the birds were kept for 11 weeks, and then for another 4 weeks at 4 % without any appreciable loss in weight.

The fifth sample of yeast extract, designated by us as *R*, was tested on a further pair of birds, starting at 1·5 % and raising the quantity to 3 % as they were not well; they died in 9 weeks. Another pair started at 4 % died in 7 weeks even after raising to 6 %. This extract thus appeared to contain very little vitamin B. The several extracts of yeast which have been so tested have varied from 3 or 3·5 to 8 or 10 % for the amount to be incorporated in a vitamin B-free diet. One extract was very weak.

Second series.

White rice soaked in marmite was not considered as an altogether suitable method of testing the value of yeast extracts, partly on account of the scattering of the food, and partly on account of the unavoidable loss of some of the extract adhering to the sides of the food containers which we used. It was decided therefore to substitute white flour for white rice. By making the flour into a dough with a solution of the marmite and making small pills about the size of a dried pea, drying the pills in the air, or at 37°, and feeding with such pills, it was impossible for the pigeons to separate the components of the food. It had to be swallowed whole, or left.

The marmite was then tested carefully at levels of 1 to 4 or more % until a quantity was reached which sufficed to maintain the birds for a long period. As seen below, the standard time of 15 weeks employed formerly by Chick and Hume [1917] is really not long enough and we have taken 20 or 26 weeks as a more satisfactory time for a maintenance test.

These experiments were made in succession as the result of a test became known. The birds on the smaller quantities showed the typical signs of polyneuritis and were allowed to die, proving the quantity was insufficient. Table X shows the results.

Table X.

Diet			Result
White flour	Fishmeal	Marmite	
90	5	2	Birds died in 7th and 10th weeks
90	5	4	„ 14th „ 20th „
90	5	5	„ 13th „ 16th „
90	5	6	„ 15th „ 18th „
90	5	8	„ 29th „ 36th „

On 2 parts of marmite the birds died with the typical symptoms of polyneuritis. One had an enlarged heart, the other showed no other special symptom.

On 4 parts of marmite, both birds showed polyneuritis. The second bird had these symptoms in the 16th week, but got better on being brought into a warm room. The symptoms appeared again in 4 weeks and the bird died. No special symptoms were noticed at autopsy.

On 5 % marmite, both birds showed polyneuritis. One bird had a distinctly large heart.

On 6 % marmite, polyneuritis was seen. The intestines were abnormal in appearance.

On 8 % marmite, the birds did not show the symptoms of polyneuritis. On *post mortem* examination the hearts were large and the intestines were abnormal.

The quantity of 8 % was taken as the minimum for long maintenance.

Third series.

It was also necessary to test the dried yeast used for the chickens as a source of vitamin B so as to compare it with the marmite.

It was being found that 6 % of dried yeast was a sufficient quantity to balance the carbohydrate for chickens. The first trial with pigeons was therefore made with 4 %.

The food was prepared as described above with white flour and made into pills.

On 4 % of dried yeast a pair of pigeons was kept for 37 weeks. During this time their weight kept constant and the hen laid six lots of eggs. The eggs were generally fertile, but only in two instances was a squab hatched; it died soon afterwards from unabsorbed yolk sac.

These birds were then given the diet with 2 % dried yeast.

They died in the 12th and 13th weeks afterwards. One bird showed the typical signs of polyneuritis; the other was paralysed. This latter bird had a distinctly large heart. The intestines of both birds were abnormal in appearance.

Other trials were made with 1 % and 3 % of dried yeast as these experiments were in progress. On less than 4 % the birds died. Still other experiments were made with larger amounts of dried yeast in the hope that rearing would be better. There was no great improvement in rearing,

but the actual hatching was better. The results of this series are seen in Table XI.

Table XI.

Diet			
White flour	Fish-meal	Dried yeast	Results
94	5	1	Both birds died in 6 weeks
93	5	2	Birds died in 12th and 13th weeks
92	5	3	Birds died in 9th and 15th weeks
91	5	4	Kept for 37 weeks. Six lots of eggs, no rearing
89	5	6	Kept 18 weeks. Five lots of eggs; 2 hatches, no rearing. Then given 8 % dried yeast
89	5	6	Kept for 48 weeks. Eleven lots of eggs; fertile, but no hatch
87	5	8	Kept 50 weeks. Five lots of eggs; fertile; no hatching
85	5	10	Kept 26 weeks. Eight lots of eggs; three hatches, but no rearing

It was clear that 4 % of dried yeast was sufficient for long maintenance and reproduction. There was no rearing. The increase of dried yeast to 10 % made some improvement in hatching. The experiment was not continued, as it was possible that some other factor than vitamin B might be concerned in the hatching of young that could be reared.

The maintenance with 4 % of dried yeast is less than the amount with chickens, which were found to need 6 % as the minimum. These results correspond with those that we have found previously, that chicks need more vitamin B than pigeons. The larger amount of dried yeast in the food is beneficial just as was noticed with the rearing of chickens. For perfect health it is advisable to give more vitamin B than the minimum required for maintenance.

Experiments with rats.

Having found that 8 % of marmite and 4 % of dried yeast were necessary to balance the food of pigeons, it was of importance to ascertain how much vitamin B in similar terms was required by rats.

Exp. 1. This experiment, though not actually the first to be started, was made partly to act as control and partly to find out how long adult rats could live on a diet free, or almost free, from vitamin B. There are numerous references to the short time of survival of young rats on such a diet, but it is difficult to find information about adult rats.

Four adult rats, one buck and three does, which had been kept previously on a diet of 90 maize, 5 marmite and 5 fishmeal for 20 weeks were given our diet of:

White flour	Fishmeal	Cod-liver oil
94	5	1

made up into pills dried at room temperature on 4. vi. 1925.

Each animal showed an increase in weight during the first 4 weeks. One of the does then had young, which died. Subsequently a decline in weight set in. The other does also had young, which were eaten by their mothers. At the 8th week, a doe again had young which were eaten. In the 9th week another doe had young during the birth of which she died. The first doe then

rapidly declined in weight and died in the 16th week. The third doe showed no signs of young.

The two remaining animals were now so ill that they were given a diet with 3 % of marmite. They showed improvement during the next few weeks, especially the buck, but they never seemed as well as our other rats. After 19 weeks on 3 % marmite in the food, both were chloroformed. The doe had seven young in her uterus, but her lungs were solid and she would have died of pneumonia. The buck was abnormal in appearance; the large intestine was yellow and contained gas; the appendices also contained gas. The animals did not appear to have recovered from the diet with no marmite.

During this period of diet with 3 % marmite, the buck was put with a doe also on 3 % marmite. This doe had been previously on 2 % marmite (see Exp. 2). At the 11th week on this diet this doe had young and she died of haemorrhage. On *post mortem* examination excess of fat in the abdominal region was the only abnormality observed.

Exp. 2. This was actually the first experiment on our white flour, fishmeal, marmite diet with 1 % of cod-liver oil. The amount of marmite was 4 %, half the amount that was found to be required by pigeons.

The object was to find out if a pair of rats could be maintained on this quantity of marmite and also reproduce. The experiment was started on 21. i. 1925 and the animals weighed 87 and 78 g. They increased in weight regularly every week. At the 6th week the doe had seven young which were eaten. At the 13th week she had a litter of nine. Five of these died, but the other four were reared by the mother and weaned in the usual time of 3 weeks (see Exp. 3).

At the 18th week the doe had another litter of nine; two of these were eaten.

As this doe had reared on 4 % marmite, the quantity was now reduced to 1 % to see if this quantity was sufficient for maintenance and rearing. The seven young gradually died, some showing distinct signs of paralysis. 1 % was thus not enough for rearing.

The two animals were then kept continuously on the diet with 1 % marmite. The doe had four more litters of young, which were at once eaten. The last litter was in the 34th week on this diet. During the birth of these young the doe died. Autopsy showed masses of fat in the abdominal region with black food residues in the large intestine extending into the small intestine. The buck rat did not look well and appeared very constipated; he passed only very few faecal pellets of very hard consistency daily. He lost weight, then gained about 40 g., then lost and then gained again. He was kept altogether 82 weeks on this diet and was then killed. Autopsy showed masses of fat in the abdominal region. Histological examination of the intestines of this rat has been made by Dr M. J. Rowlands who is publishing his results independently.

Exp. 3. The four young rats from the pair on 4 % marmite (Exp. 2) after

weaning were given the diet with 3 % marmite for the next 3 weeks. During this time they increased in weight from 110 to 303 g., average 48 g. They were one buck and three does.

The quantity of marmite was now reduced to 2 % to see if this amount was enough for maintenance and reproduction. It had been seen that 1 % was not enough.

In the 7th week on this diet, one of the does gave birth to a litter of seven. Two were eaten at once, the others gradually died in the next 2 weeks. During the next 15 weeks each of the does had several litters of young. In every case the young were eaten by the mother. One doe was then changed to 3 % marmite (see Exp. 2). The other two does were kept on the 2 % diet for altogether 25 weeks. Both had young which they immediately ate. It thus seemed that 2 and 3 % marmite were not enough for rearing the young. The diet was changed to 4 % marmite, upon which quantity young had been reared (Exp. 2), for a further trial of this quantity. One doe had seven young which she ate. The other had ten, nine of which she reared (Exp. 4). The rats were kept on this 4 % diet for 16 weeks. Both does had young, but in each case there was no rearing. One of the does then died and at autopsy was found to have a ballooned intestine.

The trials with pigeons were now showing the superiority of dried yeast over marmite in vitamin B content. The marmite was changed to dried yeast. The pair was kept on 4 % dried yeast for 17 weeks. The young that were born were eaten in every instance by the mother. The dried yeast was raised to 6 % to see if a further increase would improve rearing. The buck died of pneumonia in the 25th week. The doe was kept for another 13 weeks and was given the buck alternately with the remaining doe from Exp. 4. No young were born. The experiment was then discontinued, as it was possible that reserves of a possible vitamin E were exhausted.

Exp. 4. In order to test again the 3 % quantity of marmite in the food, the nine young rats from 4 % (Exp. 3) were placed on this food after weaning. They increased from 26 to 74 g. on the average during the first 4 weeks. There were four bucks and four does and the ninth unfortunately escaped. Only one buck was kept and he was placed alternately with the four does, which were kept two in a cage except at birth of young.

Those on 3 % did not thrive well, only increasing 50 to 60 g. in the next 5 weeks. They were found one morning accidently injured and were chloroformed.

Those on 5 % marmite gained 70 g. in the first 5 weeks. One of these does also met with an accident during one night and was chloroformed. At this time the marmite was changed to dried yeast, as the marmite seemed less good than formerly.

A litter of five was born in the 18th week and two were reared. Though the pair was kept for another 40 weeks no more young were born. Altogether the pair was kept for 61 weeks on this diet.

Exp. 5. The two young rats from the 5 % dried yeast pair were two bucks. They were put upon a diet with 1 % dried yeast to compare with the diet of 1 % marmite. They were kept for 27 weeks and were then killed. On *post mortem* examination they were found abnormally fat in the abdominal area. Histological examination was carried out by Dr Rowlands who is recording his results separately.

Rats have thus been kept on diets containing 0, 1, 2, 3, 4 % of marmite. Adults were maintained for the long period of 34 and 82 weeks on 1 %, but young could not be reared. Young were not reared on 2 and 3 % marmite, but two litters were reared on 4 % marmite by different does. 4 % thus appears as the least possible quantity for proper maintenance.

Using dried yeast instead of marmite, young rats were kept for 27 weeks on 1 %, but on examination at autopsy they were abnormal. Reproduction was poor on 5 and 6 % dried yeast.

On comparison with pigeons, 4 % marmite for a rat corresponds with 8 % marmite for a pigeon and 2 % dried yeast with 4 % dried yeast, though rats could be maintained for a long period on 1 % dried yeast. Emmett and Peacock [1923] have also observed that rats have a smaller vitamin B requirement than pigeons.

A balance of food by vitamin B is thus also shown in the case of rats.

Discussion of results.

Though our experiments on the need of balancing the protein in the diet by vitamin B are not so conclusive as was hoped, there was no doubt that the chickens and ducks on the high amount of protein in the food were in better condition with additional marmite, or dried yeast. If our observations be taken in conjunction with those of Reader and Drummond [1926] and of Hartwell [1924, 1925] on rats, the conclusion may definitely be drawn that the protein of the food must be balanced by vitamin B. The ill-effect of the high protein on birds, not noticed on rats, is most likely due to the kind of protein. Reader and Drummond and Hartwell used caseinogen and in former experiments [1922] we have used a high proportion of milk proteins with which we reared first-class birds. It is probable that the flesh (fish) protein was the cause of the abnormalities in the birds.

The need for balancing the fat in the diet by vitamin B was made quite obvious in the miserable external appearance of the birds on the high fat diet with an insufficient amount of dried yeast.

The balance of the carbohydrate, though not so obviously affecting the external appearance, was shown by the abnormal *post mortem* condition of the birds at the end of the experiment.

Our results agree with those of Randoin and Simonnet [1924] who came to the idea that the ratio

$$\frac{\text{vitamin B}}{\text{carbohydrate}} = \text{a constant},$$

but our results point to a wider relationship, such as

$$\frac{\text{vitamin B}}{\text{calories}} = \text{a constant.}$$

If reference be made to the composition of our diets (pp. 923, 925) and if the ratio which we propose be considered, then in the high fat experiments, the ratio $\frac{\text{dried yeast}}{\text{calories}} = \frac{1}{40}$

and in the carbohydrate experiments the ratio is $\frac{1}{61}$, with 6 % of dried yeast, or $\frac{1}{37}$ with 10 % dried yeast. In the former case the yeast was scarcely sufficient, but the latter figure gave birds as good as those with 16 % dried yeast on the high fat diet.

A similar figure could not be arrived at from the protein experiments, as in all the experiments the ratio was greater than $\frac{1}{40}$.

In the case of pigeons the ratio is rather less, about $\frac{1}{80}$, and in the case of rats about $\frac{1}{160}$, if 2 % of dried yeast be taken as the minimum figure for their maintenance.

Cowgill, Smith and Beard [1925] and Beard [1925] have also found a relation between vitamin B and both food consumption and the body weight. They have proposed the relation

$$K = \frac{\text{vitamin B}}{\text{calories} \times \text{weight}^{\frac{2}{3}}}$$

and consider that during growth some additional factor affects the vitamin B requirement.

We have previously [1923] observed that young pigeons cannot be reared on the same diet as that on which their parents can be maintained. They need more vitamin B. The difference corresponded with the difference between adult pigeons and chicks. Young rats again could not be reared on the same diet as that on which their parents could be kept.

The function of vitamin B is more than that of simply balancing the food consumed and bearing a relation to the body weight. Drummond and Marrian [1926] have found that its rôle is not connected with the internal metabolism of the cell, for, in its absence from the food, the condition resembles the condition of the tissues in starvation.

Vitamin B appears to be of fundamental importance in life, having a rôle not only in general metabolism, but also a special rôle in relation to the endocrine organs. We believe that vitamin B is a definite and important chemical constituent of every cell, in fact a constituent of the nucleus. Without vitamin B the nucleus cannot exist. The greater abundance of vitamin B in nuclear tissues supports such a proposition, and, if Funk's original "vitamine" and other substances which have been isolated be accepted, the nuclear connection is emphasised by the fact that these substances belong to the pyrimidine or purine groups. The recent isolation of the antiberiberi vitamin

by Jansen and Donath [1927] again includes the vitamin in this group of compounds.

The function of protein in the food is to repair so-called wear and tear of the cell and for growth in young animals. Similarly, the function of vitamin B is to replace wear and tear of the nucleus and to aid the growth of nuclear material. Hence the greater need of vitamin B by young animals, for growth of the nuclei. The difference in requirements between birds and rats may be in connection with the amount of nuclear material in the whole body. Rats may have a relatively smaller amount of nuclear matter for repair and for growth.

Since it is clear that vitamin B must bear a relation to the total food consumed, it would follow that the general idea of a daily dose or quantity is not correct. Such a quantity might be determined for a small amount of daily food, but this quantity would not suffice for a larger amount. The quantity of vitamin B required daily must be in relation to the maximum daily food consumption.

Our experiments further make a distinction between absence of vitamin B, an insufficiency and a proper amount. The term deficiency has been generally used to describe the condition of total, or nearly complete, absence. The term partial deficiency has been used for amounts between absence and the proper amount. We propose the term *shortage* for the intermediate condition of too little, more particularly to a slight degree which produces no immediate ill-effects.

Very definite pathological symptoms have been noticed in our animals kept for long periods on a shortage of vitamin B. The very large amount of fat in the abdomen was the most striking condition. The large intestine was often blocked with dark residues of food, occasionally extending into the small intestine. Sometimes there was stoppage in the large intestine, as was noticed by the impossibility of pushing the food down the alimentary canal. The pigeons often had the cloaca distended with excreta. It has been noticed also that after evacuation pigeons with polyneuritis were distinctly better. The appendices were abnormal. The hearts in many cases were obviously enlarged. The tissues were sometimes wet. The gizzards in some cases had very thin walls. Unfortunately we have not been able to study the pathology of the organs, but Dr Rowlands is referring to the histology of the intestines in cases of shortage in rats, and Dr Pulvertaft has examined many of the hearts in our dead birds.

The pathological appearances which we have noticed are the same as described by McCarrison [1921] as occurring in the early stages of beriberi, in which vitamin B is absent from the diet. The early stages of beriberi have thus become a chronic condition on a diet with a continual slight shortage of vitamin B. They are the conditions commonly observed in the population at the present time. For this reason we have also made tests of the comparative vitamin B value of the common foods, some of which we hope to have ready for publication shortly. These tests indicate that the general diet of the people

is like that of the birds on a slight shortage of vitamin B, and that the symptoms produced by vitamin B shortage are the same in birds and man. The prevalence of gastro-intestinal trouble and heart affections may be traced to a continual slight shortage of vitamin B.

One other factor may be considered as concerned in the pathological condition of our animals, namely, the fat-soluble vitamin D. Though 2 % cod-liver oil was fed to all the chickens in the last series of experiments and rats to supply fat-soluble vitamins, it is possible that owing to faulty digestion on account of shortage of vitamin B the oil was not assimilated, so that there was at the same time a shortage of vitamin D. A full supply of vitamin B is essential for the introduction into the system of the vitamins A and D in the fat of the food.

Our results and conclusions are based upon the old idea that marmite and dried yeast contained only one vitamin B. Two vitamins, which may be termed B 1 and B 2, are now believed to be present in these foods. It is therefore possible that both B 1 and B 2 are concerned, or possibly B 2 only. The future will have to decide upon the separation and differentiation of these vitamins. For practical purposes, at any rate of human dieting, the existence of the two vitamins, closely associated in yeast and other foods, is in the same category as the existence of two fat-soluble vitamins, A and D in cod-liver oil, butter, other fats and eggs. The foods which supply the one most often contain the other also, so that they are consumed together.

Summary.

Protein, fat and carbohydrate in the diet need balancing by vitamin B (B 1 + B 2). The quantity of dried yeast, which carries the vitamin B required, can be represented by

$$\frac{\text{dried yeast}}{\text{calories}} = \frac{1}{40} \text{ to } \frac{1}{60}$$

or from 6 to 10 % in a food containing no vitamin B in the case of chickens. The ratio is about $\frac{1}{80}$, or 4 % of the food, in the case of adult pigeons and $\frac{1}{160}$, or 2 %, in the case of adult rats.

8 % of marmite corresponded with 4 % of dried yeast as tested on pigeons. Samples of yeast extract have been found to vary considerably in their vitamin B content.

Young animals require more vitamin B than adult animals. It is therefore suggested that vitamin B is a constituent of the nucleus of the cell, and is required for growth and for replacement of wear and tear.

Various pathological conditions, obviously affecting the intestines and heart are noticed on diets containing too little, or a shortage, of vitamin B. These symptoms have been described as occurring in the early stages of beriberi. On a shortage of vitamin B these early symptoms become a chronic condition of ill-health.

We gratefully acknowledge a grant from the Government Grant Committee of the Royal Society, which has covered a portion of the expenses of these experiments. We also wish to thank the Marmite Food Extract Co., Ltd., for kindly placing at our disposal marmite and dried yeast.

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CXXVI. EXPERIMENTS ON NUTRITION. VII.

FAT-SOLUBLE VITAMIN REQUIREMENTS OF CHICKENS.

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Our original objects in the study of the rearing of chickens were to ascertain the minimum quantities of each vitamin, in terms of cod-liver oil, marmite or yeast, and orange juice, that were required [1922]. We have found [1923, 1] that vitamin C is not required, that the vitamin B requirement is 9 % of marmite [1923, 2], or 10 % of dried yeast [1927]. The quantity of fat-soluble vitamin in terms of cod-liver oil has in the meantime been determined by Dunn [1924] at 0·5 %. Emmett and Peacock [1923] have also referred to the requirement of vitamin A by chickens and contrasted their requirement with that of pigeons and rats. Pigeons need very little, if any, and rats were thought to need less than chickens.

It became necessary for us, in connection with our work on the balance of food by vitamin B, to ascertain the amount of fat-soluble vitamins required, since a possible cause for the bow legs and other deformities observed on a high protein diet was little cod-liver oil in the diet.

Experimental.

These trials have been made during the course of the previous experiments. The conditions of rearing were the same as then described.

All the cod-liver oil has been obtained from the same source and said to be of the same quality. It was procured in 1 or 2 gallon lots at different times and may not always have contained the same amount of fat-soluble vitamins. This may account for slightly different results with approximately the same quantity of the oil. It was not possible to test each lot for its vitamin content by special experiments.

A series of experiments, as shown in Table I, has been made.

The first three experiments were made in the same fashion as the first three series with high protein (see foregoing paper), in which one part of cod-liver oil was made up with the food. Group XXIII is the corresponding one to those in this series. The last five experiments were made with a diminishing percentage of cod-liver oil. Groups XXXIII and XXXV are controls.

Table I.

Group	White rice	Dried yeast	Fishmeal	Cod-liver oil	No. of birds	Losses
XXIV	90	20	5	0	8	8
XXXI A	90	20	5	0·5	3	2
XXXII A	90	20	5	0·7	8	4
			or			
XXIV	78	17	4	0		
XXXI A	78	17	4	0·4		
XXXII A	78	17	4	0·6		
XXXIX	75·3	16	8	0·7	8	0
XLIII	75·5	16	8	0·5	9	0
XXXI B	75·7	16	8	0·3	8	1
XXXII B	75·8	16	8	0·2	8	1
XLVIII	75·9	16	8	0·1	7	2

Group XXIV. All the birds died within 4 weeks. Autopsy showed that the bones were soft. The intestines showed no special abnormality, but the kidneys were generally pale in colour. In one bird the body cavity was full of water.

Group XXXI A. The birds were clumsy on their legs in the 3rd week; the legs were stretched outwards rather than twisted as in the abnormalities on the high protein diets. The two losses were in the 4th and 5th weeks. The yolk sacs were found not to be completely absorbed. The livers were not normal and the kidneys seemed distended. The excretion of these birds seemed difficult. The remaining bird was then placed in the group XXXII A.

Group XXXII A. The four losses occurred in the 3rd and 4th weeks. All these birds showed some congestion round the intestines; the guts were clean. The lungs of all were congested and they died of pleurisy. The other birds seemed fairly normal. The experiment was not continued further, as it already indicated that 0·6 part of oil was hardly sufficient. The early deaths of these birds in contrast with the longer life of the birds in the protein series is remarkable. The slightly larger quantity of cod-liver oil in those experiments was at any rate beneficial, though it may not have been enough. The later experiments were made to compare with the fourth series of protein experiments and the series with high fat and carbohydrate.

Groups XXXIX *and* XLIII. The birds in these two groups were probably the best birds that were reared in any of our experiments. Those of group XLIII with one cock were kept altogether for about 40 weeks and never showed any signs of illness, or deformity. They started laying in the 24th week. Many of the first eggs were soft, and the birds were egg eaters. On testing for fertility by incubation the eggs were mostly unfertile.

0·5 % of cod-liver oil of good quality is sufficient to rear chicks to maturity.

The other experiments in this series were made to find out how far the quantity of cod-liver oil could be reduced.

Group XXXI B with 0·3 % of oil gave a good lot of birds with only one loss. This bird was weak on its legs in the 12th week and was killed in the 19th week. There was no fat inside, but the appendices were slightly swollen; no sign of gut stoppage could be found. The gut was practically empty; the

heart was possibly a little larger than normal. Examination of the birds at the end of the experiment when they were killed showed the intestines to be practically normal; the appendices were not quite normal in all the birds; in one case there was dark food material in the appendices. The hearts seemed slightly large.

Group XXXII B. The one loss was in the 9th week. This bird was weak on its legs and was chloroformed. It had a large wound in one leg, probably from injury. No abnormality could be seen internally. Two of the birds had deformed wings, but no special error could be detected in the internal organs after death. There was a little fat round the gizzards, in one case there was fat round the heart.

Group XLVIII. The first loss was in the 5th week. The kidneys were very pale. The appendices were distended and there was blood on the food residues in them. The rest of the gut seemed normal. The other loss was in the 15th week. This bird was shaky for the previous 2 weeks and had to be killed. The intestines seemed normal, though dark food material was present in the appendices.

It was remarkable that the birds could be reared on such small quantities of cod-liver oil as 0·1 and 0·2 %. In appearance, however, the birds could not be said to be quite fit and well.

A comparison of the weekly weights (g.) of the different groups, given in Table II, shows that the rate of growth is almost the same in the several groups. The cocks grew more slowly on quantities of cod-liver oil below 0·5 %, as was made evident first at the 10th and 11th weeks.

Table II.

	Cocks				
Group ...	XLVIII	XXXII B	XXXI B	XLIII	XXXIX
Cod-liver oil %	0·1	0·2	0·3	0·5	0·7
Week					
0	41	38	38	41	39
1	73	56	54	61	68
2	117	82	86	102	128
3	183	148	140	176	211
4	272	236	192	215	299
5	325	348	273	315	384
6	407	502	357	442	502
7	550	653	461	552	623
8	678	810	650	726	827
9	833	993	847	956	963
10	995	1085	957	1135	1181
11	1155	1290	1231	1362	1401
12	1388	1485	1363	1621	1682
13	1527	1540	1611	1817	1856
14	1755	1847	1808	2074	2105
15	2082	2010	1988	2314	2304
16	2068	2235	2185	2520	2586
17	2372	2505	2249	2794	2730
18	2618	2547	2537	2794	2910
19	2892	2805	2735	3066	3045
20	2972	2800	2974	3007	3348
21	3075	2745	—	3112	3485

	Hens				
Group ...	XLVIII	XXXII B	XXXI B	XLIII	XXXIX
Cod-liver oil %	0·1	0·2	0·3	0·5	0·7
Week					
0	40	39	39	37	38
1	71	52	55	56	70
2	119	78	88	92	117
3	182	124	137	154	175
4	294	187	185	187	243
5	298	269	275	260	305
6	383	361	357	372	402
7	490	476	445	477	500
8	593	588	610	615	637
9	732	745	790	796	768
10	865	855	878	939	915
11	1033	1009	1085	1079	1050
12	1233	1178	1162	1263	1238
13	1373	1275	1330	1404	1300
14	1622	1501	1432	1549	1455
15	1687	1587	1548	1684	1578
16	1712	1765	1690	1810	1737
17	1872	1903	1748	1950	1807
18	2025	1914	1960	1954	1927
19	2173	2117	2095	2161	2000
20	2236	2177	2130	2142	2122
21	2370	2030	—	2259	2220

The earlier experiments showed that 0·4 and 0·6 % of cod-liver oil were too little. The later experiments showed that 0·5 % was enough. The actual quantity depends upon the actual value of the cod-liver oil. In general it may be said that 1 % will be an ample quantity.

Experiments on a maize diet.

Some work in another direction using maize as a source of carbohydrate and vitamin B has led to another test regarding the requirement of cod-liver oil in the diet. There have been three groups tried so far for this purpose.

Diet.

Group	Maize	Fishmeal	Cod-liver oil	No. of birds	Losses
M V	89	10	1	8	0
M VI	90	10	0	9	5
M VII	89·75	10	0·25	6	1

M V birds have been reared successfully.

M VI birds showed definitely in the first 4 weeks symptoms of rickets and five died, showing congested intestines and appendices with stiff pasty food. These birds are shown in Plate VI, fig. 6.

Their appearance is quite different from that of the birds on high protein diet. Their legs are stretched outwards. The high protein birds had either bow legs or were knock-kneed. Often also the birds with too little vitamin B had a twisted leg. The outstretching seems to be the sign of rickets in chicks. In the early symptoms of polyneuritis the bird tends rather to sit on its legs and one leg tends to bend underneath the bird.

M VII birds, except one, have lived for 8 weeks and up to that time have shown no signs of rickets in their legs. Signs have then appeared.

It is thus essential to add cod-liver oil to the maize diet to rear chicks, and 0·25 % is too little.

Just as in the rice-dried yeast birds the minimum quantity of cod-liver oil appears to be 0·5 %.

We have several times observed that on keeping groups of birds beyond maturity these small quantities of cod-liver oil are not enough. The eggs have been unfertile and if fertile did not hatch, or the chicks soon afterwards died. On raising the cod-liver oil to 3 % improvement in fertility and hatching was observed. It is proposed at a later date to test this point specially.

SUMMARY.

The quantity of cod-liver oil that is required for rearing chicks is 0·5 % as a minimum. Samples of cod-liver oil, presumably of the same quality, vary and it is better to take 1 % as the smallest quantity.

Part of the expense of this work has been defrayed out of a grant to one of us from the Government Grant Committee of the Royal Society, to whom we express our thanks. Dried yeast has been kindly placed at our disposal by the Marmite Food Extract Co., Ltd.

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CXXVII. THE DIURNAL VARIATION OF THE GASEOUS CONSTITUENTS OF RIVER WATERS.

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Historical introduction.

Morren [1841] studied the variations of dissolved oxygen in an aquarium throughout almost a year and made observations of diurnal changes of the oxygen content of the water. His figures, however, record the percentage oxygen in the total dissolved gases, and he makes only one reference to the actual amount present. He says: "Le volume de l'air dissous était variable et augmentait notablement avec la quantité d'oxygène. La quantité d'azote restait sensiblement constante mais l'acide carbonique contenu dans l'eau variait aussi." He gives no figures of actual quantity present, but it is of interest that he records supersaturation.

In rivers, however, he finds no such variation. He suggests that his failure to detect a maximum is due to a rapid current, and a sandy bed, and without giving any figures says that a slow river flowing over mud does not contain so much dissolved oxygen, and that the figures obtained show considerable variation.

Considerable work has been done on the seasonal variations of dissolved gases in lakes and reservoirs, and on the relation of the dissolved gases to the presence of algae.

Birge and Juday [1911] have recorded the dissolved oxygen content of Lake Mendota at various depths over a period of 12 months, but showed no diurnal variation.

Butler and Coste [1923], working in the Thames estuary, showed seasonal variation of dissolved oxygen, and commented on the phenomenon of supersaturation.

The relation of dissolved gases to algae has been demonstrated by Chambers [1912] on the results of his work in the lagoons at the botanical gardens of St Louis; of special interest is his observation that on clear sunny days the water became supersaturated with dissolved oxygen, while on cloudy days the percentage of oxygen fell below saturation, sometimes as low as 40 %.

Moore [1921], correlating the results of 10 years' work, showed the seasonal variations and the interrelations of dissolved oxygen, diatoms, light, temperature, nitrite and alkalinity in the sea.

The present paper giving the diurnal variation of dissolved gases in small rivers is an addition to the data there recorded.

Cowles and Schwitalla [1923] have recorded some similar variations in hydrogen ion concentration, but no figures have been given for diurnal variation in ammoniacal nitrogen.

Observations on river waters.

During the past year several surveys have been made of the River Lark in connection with the pollution by the effluents from the beet-sugar factory at Bury St Edmunds. The River Lark is a small river rising in the chalk hills to the south of Bury St Edmunds. From here it flows in a north-westerly direction and joins the Great Ouse near Prickwillow, 25 miles from the source.

The beet-sugar factory at Bury St Edmunds discharges its waste waters into the river about a mile north of the city, and the part of the river under observation stretches from Bury through the villages of Fornham, Lackford, Icklingham, Barton Mills, Mildenhall to Westrow, a distance of 14 miles. The usual determinations made on these surveys were:

1. Dissolved oxygen.
2. Ammoniacal nitrogen.
3. p_H value.
4. Water temperature.

It was noticed that occasional anomalies occurred in the values obtained from the dissolved oxygen determinations, *e.g.*

At Icklingham, July 29th, 1926, 10.20 a.m., 59 % of saturation of O_2.
2.5 p.m., 105 ,, ,,
At Fornham, March 22nd, 1927, 3.35 p.m., 112 % of saturation of O_2.
23rd, 1927, 4.20 p.m., 88 ,, ,,
24th, 1927, 12.20 p.m., 118 ,, ,,
25th, 1927, 11.20 p.m., 90 ,, ,,

and it was concluded that such factors as time of sampling, weather conditions, amount of light, etc., have a greater effect upon the amount of dissolved oxygen present than was previously supposed.

River Lark. Series I.

Accordingly, it was decided to carry out hourly determinations of dissolved oxygen, ammoniacal nitrogen, p_H and water temperature, over a period of 24 hours at a fixed point.

A convenient place for sampling was chosen at Icklingham, 7 miles below the confluence of the river and the leet from the beet-sugar factory.

The figures obtained over a period of 10 months are given below to indicate the changes produced at this spot during the beet-sugar campaign.

Table I.

Date	Time	Temp. ° C.	Dissolved oxygen. Parts per 100,000	% saturation of oxygen	p_H value	Ammoniacal nitrogen. Parts per 100,000	Remarks
29. vii. 1926	10.20 a.m.	15	0·59	59	7·5	0·05	
29. vii. 1926	2.5 p.m.	17	1·01	105	—	—	
14. ix. 1926	7.20 p.m.	15	1·05	102	8·2	0·02	
6. x. 1926	—	14·5	—	86	8·0	0·01	Fermented effluent discharged from factory
27. x. 1926	5.15 p.m.	6	0·395	31	7·5	0·02	Unfermented effluent discharging from factory
28. x. 1926	2.5 p.m.	6	0·485	38	7·5	0·02	
29. x. 1926	10.15 a.m.	7	0·203	17	7·4	0·15	
16. xi. 1926	3.10 p.m.	9·5	0·096	8	7·3	0·01	Process water and wash water discharging from factory
17. xi. 1926	2.10 p.m.	10	0·090	8	7·3	0·015	
23. ii. 1927	5.45 p.m.	9	0·702	60	7·6	0·03	Effluent ceased, but pits being washed out
24. ii. 1927	9.45 a.m.	8	0·602	52	7·6	0·08	
25. ii. 1927	4.45 p.m.	6	0·845	67	7·8	0·02	Full flood
7. iii. 1927	5.45 p.m.	7	1·021	83	7·7	0·04	
8. iii. 1927	4.35 p.m.	8	1·098	91	7·8	0·035	
10. iii. 1927	1.45 p.m.	6·5	1·112	90	7·7	0·04	
22. iii. 1927	2.15 p.m.	12	1·588	144	7·8	0·02	
23. iii. 1927	3.10 p.m.	12	1·555	141	8·0	0·015–0·02	
25. iii. 1927	12.15 p.m.	8	1·221	102	7·8	0·03	
26. iv. 1927	11.40 a.m.	8·75	1·354	118	7·7	0·035	

From the above figures it will be seen that, at this spot during the months of October and November, 1926, the river was badly de-oxygenated when the various effluents were being discharged from the factory, and that, after February, 1927, when the beet-sugar campaign ended, the recovery in oxygenation was rapid.

As a result of the October and November pollution, the whole bed of the river over a stretch of 10 miles had been covered with a growth of *Sphaerotilus natans*, a sewage fungus, which in February, 1927, commenced to turn brown, decay and break up.

By March 11th, the sewage fungus had almost disappeared and on the date of the experiment, March 30/31st, large quantities of diatoms had grown on the weeds and river bottom (see below). At Icklingham, the river is about 30 feet wide, and has an average depth of 3–4 feet, and 200 yards below the point of sampling is a mill where the river is dammed. The speed of flow is about 600 yards per hour.

The period of observation extended from 7 p.m., March 30th to 7 p.m., March 31st; hourly samples were taken, and the following determinations made:

1. Dissolved oxygen (Winkler's method).

2. Ammoniacal nitrogen (by direct nesslerising and subsequent comparison against artificial colour standards).

3. p_H value. (On the River Lark, by addition of cresol red or phenol red and comparison against Clarke's colour chart. On the River Itchen, by Gillespie's method.)

4. Water temperature.

The results obtained are given in Table II and graphically in Fig. 1.

Table II.

Time G.M.T. Mar. 30th, 1927	Temp. °C.	Dissolved oxygen. Parts per 100,000		% saturation dissolved oxygen		Ammoniacal nitrogen. Parts per 100,000	p_H value	Remarks
		Without prelimin. oxidation	With prelimin. oxidation	Without prelimin. oxidation	With prelimin. oxidation			
7 p.m	9·4	1·384	1·377	119	119	0·01–0·015	7·9	Fine
8 ,,	9·2	1·260	1·249	108	107	0·015	7·9	,,
9 ,,	9·2	1·147	1·127	98·5	97	0·02	7·9	,,
10 ,,	8·6	1·036	1·017	88	86	0·025–0·03	7·9	,,
11 ,,	8·0	0·972	0·711	81	59	0·03–0·035	7·8–7·9	,,
Midnight Mar. 31st, 1927	8·0	0·865	0·846	72	70·5	0·11	7·7–7·8	
1 a.m.	7·7	0·798	0·784	66	65	0·13	7·7–7·8	
2 ,,	7·1	0·797	0·796	65	65	0·16–0·17	7·6–7·7	
3 ,,	7·1	0·795	0·694	65	56	0·15	7·6–7·7	
4 ,,	7·0	0·800	0·805	65	65	0·15–0·16	7·6–7·7	
5 ,,	6·75	0·806	0·791	65	64	0·16	7·6–7·7	Dawn
6 ,,	6·9	0·808	0·769	66	63	0·13–0·14	7·6–7·7	Bright
7 ,,	6·9	0·810	0·783	66	63	0·1	7·6–7·7	Bright sunlight
8 ,,	6·9	0·838	0·861	68	70	0·07	7·6–7·7	,,
9 ,,	7·0	0·953	0·920	77	75	0·06	7·6–7·7	,,
10 ,,	7·5	1·094	1·089	90	90	0·07–0·08	7·7	,,
11 ,,	8·2	1·413	1·405	119	118	0·03	7·8	,,
Midday	8·9	1·535	1·501	131	129	0·02	7·8–7·9	Dull
1 p.m.	9·25	1·679	1·646	144	141	0·01–0·015	7·9	,,
2 ,,	9·5	1·758	1·692	151	146	0·01–0·015	7·9–8·0	,,
3 ,,	10·0	1·803	1·819	157	158	0·01	7·9–8·0	,,
4 ,,	10·0	1·767	1·726	153	150	0·01	7·9–8·0	,,
5 ,,	9·75	1·690	1·614	146	139	0·01	7·9–8·0	Rain
6 ,,	9·6	1·513	1·467	130	126	0·01	7·9–8·0	,,
7 ,,	9·25	1·343	1·270	116	109	0·01–0·015	7·9–8·0	Sunset

Sunset March 30th, 1927, 6.28 p.m.
Sunrise ,, 31st, ,, 5.40 a.m.
Sunset ,, 31st, ,, 6.29 p.m.

Variation of dissolved oxygen.

The dissolved oxygen content of the river showed a remarkable periodic variation between 0·795 and 1·803 parts per 100,000, *i.e.* between 65 and 157 % of saturation of oxygen at the temperature of sampling.

From 7 p.m., March 30th, the values obtained decreased steadily until 1 a.m., March 31st, from 1 a.m. to 7 a.m. the dissolved oxygen content was at a minimum, after which a very rapid increase took place, reaching the maximum value at 3 p.m.; from 3 p.m. onwards, a rapid decrease took place for the remainder of the period of observation.

From 9 p.m., March 30th, until 10 a.m., March 31st, all samples had a dissolved oxygen content below 100 % of saturation, while for the remainder of the 24 hours the water was supersaturated.

The samples which underwent preliminary oxidation before estimation of dissolved oxygen generally gave values about 2 % less than those fixed and estimated in the ordinary way.

Variation of ammoniacal nitrogen.

Considering the fact that the ammoniacal nitrogen content of a normal river water is about 0·01 parts per 100,000, the figures obtained during this experiment must be regarded as abnormal, and it may here be pointed out that very high ammonia figures have been obtained at various stations on this river, coincident with the presence of large quantities of broken and decaying sewage fungus.

At 7 p.m., March 30th, the water contained 0·01–0·015 parts N per 100,000, which increased to 0·015 parts at 8 p.m. From 8 p.m. until 11 p.m. the increase in the ammoniacal nitrogen content was more rapid, and after 11 p.m. it increased still more rapidly, reaching a maximum value of 0·165 parts per 100,000 at 2 a.m., March 31st, and remaining at about this figure until 5 a.m.

After 5 a.m. the values obtained fell very rapidly until 1 p.m.; the minimum value of 0·01 parts per 100,000 was reached at 3 p.m. and this figure was maintained until 6 p.m., followed by a slight increase at 7 p.m.

Variation of p_H value.

During the period of observation the reaction of the water changed slightly between p_H 7·6 and 8·0.

After 10 p.m., March 30th, the p_H value decreased gradually, reaching a minimum at 2 a.m., March 31st, and remaining constant until 9 a.m., after which a steady increase took place to a maximum value at 2 p.m. which persisted until 7 p.m.

Variation of water temperature.

From 7 p.m., March 30th onwards, the water temperature fell steadily, reaching a minimum of 6·75° at 5 a.m., March 31st. After 10 a.m. it increased constantly to a maximum of 10° at 3 p.m., after which a steady decrease took place. The range was from 6·75° to 10°.

Conclusions based on the chemical data.

It is well known that all green plants under the influence of light consume carbon dioxide and liberate oxygen. On the date of the experiment, the river contained very few of the larger green plants, but diatoms were at the spring maximum of their growth. The result is seen in the abnormal figures of supersaturation of dissolved oxygen obtained during the experiment. Large clots of these organisms, floating in the water, gave evidence of their abundance. Rough estimations of this growth have been made by putting glass slides on the river bed. The slides are each 2·5 × 8·2 cm. and four are fixed in an ordinary photographic printing frame. These are taken up after a fortnight's immersion, washed with distilled water and the deposit dried at 100° and weighed.

	Amount in g. per sq. cm.	
Dates of immersion	No. 1 frame	No. 2 frame
1. xi. 1926 to 15. xi. 1926	0·003	0·004
9. iii. 1927 to 23. iii. 1927	0·010	0·015
23. iii. 1927 to 7. iv. 1927	0·073	0·070
7. iv. 1927 to 21. iv. 1927	0·047	—

Animals and fungi, as well as green plants, take up oxygen during both day and night and give out carbon dioxide. During the night, photosynthetic production of oxygen ceases, while biological and chemical consumption of oxygen continues, so that the dissolved oxygen content of the water is at its minimum during this period.

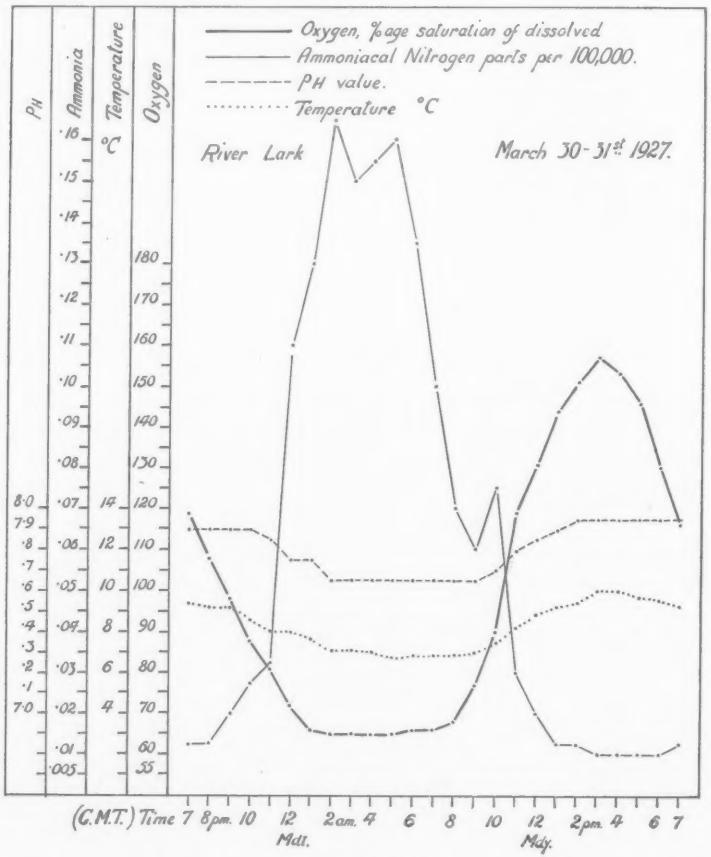

Fig. 1.

At Lackford (2 miles above Icklingham) a small sewage effluent enters the river with an ammoniacal nitrogen content of 0·8 parts per 100,000. Previous samples, taken during the day, 400 yards below the confluence of the leet and river, show that the dilution is sufficient to reduce the nitrogen content to 0·01 parts per 100,000.

By the decay of vegetable matter a considerable amount of ammonia is produced; some figures, obtained on previous surveys, are given below showing

very high ammonia values coincident with the presence of large quantities of broken sewage fungus.

	N parts per 100,000
Feb. 23rd, 1927, Barton Mills	0·095
„ 23rd, 1927, Mildenhall	0·135
„ 23rd, 1927, Westrow	0·125
Mar. 7th–10th, 1927, Fornham to Westrow (15 miles)	0·03–0·15
„ 22nd, 1927, Barton Mills	0·16

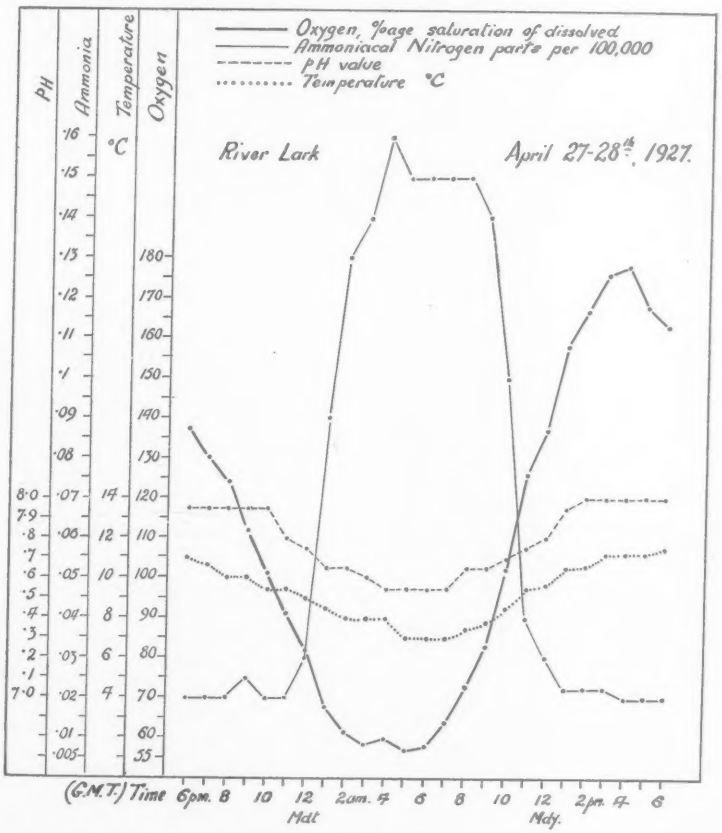

Fig. 2.

During the day, when the oxygen content of the river is high, the ammonia is oxidised to nitrates, with the result that after 3 p.m., when the oxygen content is at a maximum, the ammoniacal nitrogen content is at a minimum [cf. Bonazzi, 1919]. Correspondingly, when the oxygen content has fallen below 100 % of saturation, less of the ammonia produced is removed by oxidation, and during the minimum of oxygen period, 1 a.m. to 7 a.m., the free ammonia figures are at a maximum.

Another possible cause of the rapid increase of free ammonia with the coming of night is that the fixation of nitrogen from ammonia may cease with the disappearance of sunlight.

During the day the photosynthetic reactions of the plants remove CO_2; at night, when photosynthesis ceases, carbon dioxide is still being liberated by plant and animal respiration with the result that a certain amount of normal carbonate is converted to bicarbonate and the p_H value of the water falls to its minimum during this period.

River Lark. Series II.

On April 27/28th, at Icklingham, a further set of observations over a period of 24 hours was made confirming the results obtained on March 30/31st. The figures are given in Table III and graphically in Fig. 2.

Table III.

Time G.M.T.	Temp. ° C.	Dissolved oxygen. Parts per 100,000	% saturation dissolved oxygen	Ammoniacal nitrogen. Parts per 100,000	p_H value	Remarks
6 p.m.	11	1·532	137	0·02	7·9–8·0	Bright sunshine
7 „	10·6	1·463	130	0·02	7·9–8·0	Good light. Sundown
8 „	10	1·422	124	0·02	7·9–8·0	Dusk
9 „	10	1·291	112	0·025	7·9–8·0	Dark. Fine
10 „	9·5	1·177	101	0·02	7·9–8·0	Clear and starry. No moon. Fine
11 „	9·5	1·058	91	0·02	7·8	„ „ „
Midnight	9	0·960	82	0·03	7·7–7·8	„ „ „
1 a.m.	8·5	0·808	68	0·09	7·6–7·7	„ „ „
2 „	8	0·738	61·5	0·13	7·6–7·7	„ „ „
3 „	8	0·702	58·5	0·14	7·6	Sky just lightening. Fine
4 „	8	0·710	60	0·16	7·5–7·6	
5 „	7	0·708	57	0·15	7·5–7·6	
6 „	7	0·723	58	0·15	7·5–7·6	
7 „	7	0·792	64	0·15	7·5–7·6	
8 „	7·5	0·894	73	0·15	7·6–7·7	Bright sunshine. Fine
9 „	7·8	1·005	83	0·14	7·6–7·7	„ „
10 „	8·5	1·212	102	0·1	7·7	„ „
11 „	9·5	1·459	126	0·04	7·7–7·8	„ „
Midday	9·7	1·580	137	0·03	7·8	„ „
1 p.m.	10·5	1·787	158	0·02–0·025	7·9–8·0	„ „
2 „	10·6	1·889	167	0·02–0·025	8·0	„ „
3 „	11·2	1·969	176	0·02–0·025	8·0	„ „
4 „	11·2	1·995	178	0·02	8·0	Cloudy. Fine
5 „	11·2	1·863	168	0·02	8·0	„ „
6 „	11·5	1·807	163	0·02	8·0	„ „

Sunset April 27th, 7.15 p.m.
Sunrise „ 28th, 4.40 a.m.
Sunset „ 28th, 7.16 p.m.

April 27th was a bright sunny day, following several bright days, whereas on March 30th the morning, although fine, was not so bright and the afternoon was dull. The effect of this difference in the weather conditions is seen in the greater difference between the maximum and minimum values of percentage saturation of oxygen, viz.:

March 30th	Maximum	157 % at 3 p.m.
April 27th	Maximum	178 % at 5 p.m.
March 30th	Minimum	65 % from 1 a.m. to 7 a.m.
April 27th	Minimum	57 % from 4 a.m. to 7 a.m.

The effect of the longer period of light on April 27/28th, compared to that on March 30/31st, is seen in the shorter period of minimum of oxygen during the night. By superimposing the March and April curves these differences are very clearly shown.

River Itchen. Series I.

For the purposes of comparison a similar experiment was made on the River Itchen in Hampshire. The general topography of this stream has been previously described [Butcher, 1927]. The Itchen is not polluted in any way. The place of sampling was immediately above a small weir at the Ministry of Agriculture and Fisheries' Research Station, Alresford, where the river is about 2 feet deep and 20 feet wide, and its speed of flow is about 1300 yards per hour.

The results are given in Table IV and graphically in Fig. 3.

Table IV.

Time G.M.T.	Temp. ° C.	Dissolved oxygen. Parts per 100,000	% % saturation dissolved oxygen	Ammoniacal nitrogen. Parts per 100,000	p_H value	Remarks
9 p.m.	9·4	1·050	90·5	<0·01	8·0	Dark
10 „	9·0	1·053	90	<0·01	7·8	Clouds obscuring moon
11 „	8·6	1·024	87	<0·01	8·0	
Midnight	8·0	1·027	86	<0·01	7·8	Moonset
1 a.m.	8·0	0·979	82	<0·01	7·7	
2 „	7·8	0·995	82	<0·01	7·8	
3 „	7·5	1·035	85	<0·01	7·6	Raining
4 „	7·5	0·978	80·5	<0·01	7·5	„ Sky just lightening
5 „	7·3	0·974	80	<0·01	7·6	„ Dawn
6 „	7·1	0·985	81	<0·01	7·5	„
7 „	7·1	1·040	85	0·01	7·5	„
8 „	7·1	1·095	90	0·0015	7·5	„ River rising and turbid
9 „	7·1	1·135	93	0·02–0·025	7·6	„
10 „	7·0	1·135	93	0·01	7·6	„
11 „	7·0	1·199	97	0·01	7·5	„ slightly
Midday	7·3	1·321	108	0·015	7·4	„ „
1 p.m.	7·4	1·322	109	0·01	7·6	„ „
2 „	7·8	1·349	112	<0·01	7·6	Fine. Dull
3 „	8·0	1·247	104	<0·01	7·6	Brighter. No sun, cold wind
4 „	8·1	1·280	107	<0·01	7·6	Fine. Storm at 4.30
5 „	8·2	1·313	109	<0·01	7·5	Fine
6 „	8·0	1·279	107	<0·01	7·6	Sun shining
7 „	8·0	1·121	93	<0·01	7·6	Sunset
8 „	7·5	1·075	88	<0·01	7·5	Clear night
9 „	7·0	0·994	81	<0·01	7·5	„

Sunset April 6th, 6.39 p.m.
Sunrise „ 7th, 5.25 a.m.
Sunset „ 7th, 6.41 p.m.

Variation in dissolved oxygen.

The dissolved oxygen curve is similar to that obtained from the Lark figures. The variation is less, ranging from 0·974 parts per 100,000 to 1·349 parts per 100,000 compared to 0·795 and 1·803 for the Lark.

Variation in ammoniacal nitrogen.

The ammoniacal nitrogen content is normally less than 0·01 parts per 100,000. It seems probable that the higher values of 0·01 to 0·025 obtained between 7 a.m. and 1 p.m. are due to the washing in of ammonia compounds from the soil by flood-water. There is in the Itchen nothing comparable to the diurnal variation of ammonia in the Lark.

Variation in p_H.

The maximum p_H value, 7·8–8·0, was obtained from 9 p.m. until 1 a.m. after which a rapid decrease was observed to 7·5–7·6 at 3 a.m., the reaction remaining constant at this figure for the remainder of the period of observation.

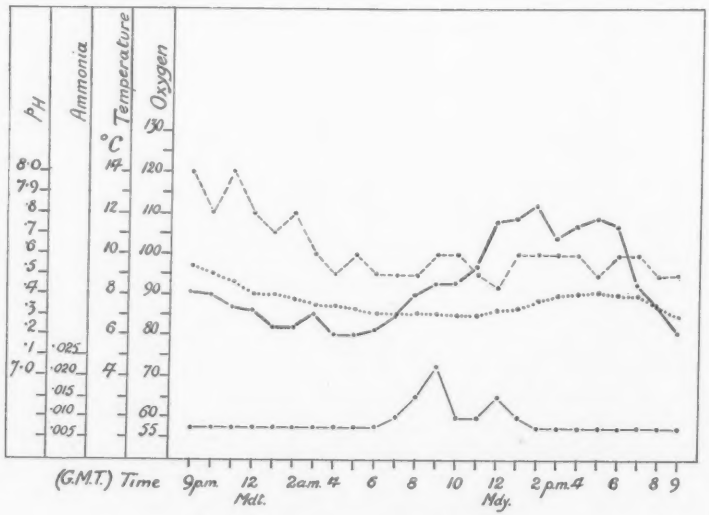

Fig. 3. River Itchen. April 6/7th, 1927.

Oxygen, percentage saturation of dissolved
Ammoniacal nitrogen, parts per 100,000
p_H value
Temperature °C

Variation in temperature.

The temperature curve showed a minimum from 6 a.m. to 11 a.m., after which it increased steadily to a maximum at 5 p.m.

River Itchen. Series II.

A further set of observations was made on the Itchen on May 4/5th and the results are given in Table V and graphically in Fig. 4.

Table V.

Time G.M.T.	Temp. ° C.	Dissolved oxygen. Parts per 100,000	% saturation dissolved oxygen	p_H value	Remarks
6 p.m.	11·6	1·170	105	8·0	Cloudy. Fine
7 „	11·3	1·043	93	7·8	Dull. Raining
8 „	11·0	0·974	87	7·7	Dusk. Dull. Raining
9 „	11·0	1·000	89	7·6	Dark. Raining
10 „	10·8	0·935	83	7·6	Cloudy. Fine
11 „	10·6	0·939	83	7·55	„ „
Midnight	10·3	0·897	79	7·55	„ „
1 a.m.	10·1	0·886	77	7·6	„ „
2 „	10·0	0·885	77	7·55	„ „
3 „	10·0	0·882	77	7·55	Sky just lightening. Cloudy. Fine
4 „	10·0	0·875	76	7·55	Dawn. Cloudy. Fine
5 „	10·0	0·893	78	7·5	Sunrise. Cloudy
6 „	9·9	0·949	83	7·55	Bright
7 „	9·9	1·035	90	7·5	Cloudy. Fine
8 „	10·0	1·135	99	7·6	„ „
9 „	10·1	1·253	109	7·55	„ „
10 „	10·4	1·170	103	7·7	Heavy thunderstorm at 9.45
11 „	10·2	1·170	102	7·6	Very dull. Raining
Midday	10·3	1·214	106·5	7·6	Raining
1 p.m.	11·2	1·285	115	7·75	Sun shining
2 „	12·8	1·409	130·5	8·0	„
3 „	13·2	1·302	122	8·0	„
4 „	13·7	1·299	122	7·9	„
5 „	13·8	1·325	124	7·9	„
6 „	13·6	1·255	118	7·9	„

Sunset May 4th, 7.26 p.m.
Sunrise „ 5th, 4.27 a.m.
Sunset „ 5th, 7.27 p.m.

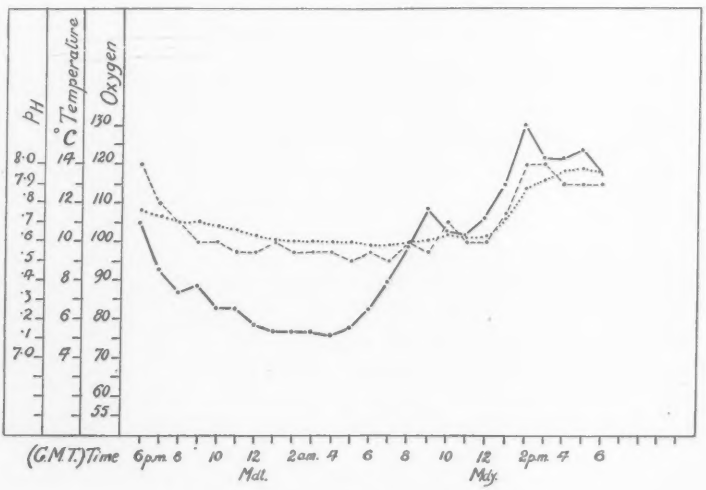

Fig. 4. River Itchen. May 4/5th, 1927.

——————— Oxygen, percentage saturation of dissolved

— — — — — — — p_H value ..... Temperature °C.

Variation in dissolved oxygen.

This curve shows greater similarity to those obtained during the Lark experiments. The greater difference between the maximum and minimum values, 1·409 and 0·875 parts per 100,000 respectively, is due to the brighter day of May 5th compared with April 7th. The irregularity in the oxygen curve after 9 a.m. was coincident with a heavy thunderstorm, the effect of which is also shown on the p_H and temperature curves.

Variation in p_H.

The p_H dropped gradually from 8·0 at 6 p.m. to 7·5 at 11 p.m., at which value it remained constant until 9 a.m. when it began to rise, was checked by the storm, and then went on to 8·0 at 3 p.m. After 4 p.m. a slight fall began.

Variation in temperature.

The temperature shows a regular fall from 11·6° at 6 p.m. to 9·9° at 7 a.m., after which a regular rise, again checked by the storm, to 13·8° occurred.

Variation in ammonia.

As in the experiment of April 6/7th, the ammoniacal nitrogen content showed no significant variation comparable to that obtained on the River Lark.

Discussion of results.

Dissolved oxygen curves.

All four curves show a similarity of structure with a minimum during darkness and a maximum 2 or 3 hours after noon.

Owing to the continuous absorption of oxygen by all organisms during the night, it seems that the oxygen content should fall continuously to a minimum value at dawn. In practice each curve shows a minimum *period* extending over several hours of the night, and it seems probable that an equilibrium is established between solution of oxygen from air and absorption of oxygen by organisms. The time-lag after dawn and after noon is in accordance with the known facts of photosynthesis [Stiles, 1925].

It will be noticed that the difference between the maximum and minimum values of oxygen obtained in the River Lark is considerably greater than that in the case of the Itchen and a comparison of the physical conditions of the two rivers is made below.

River Lark	*River Itchen*
Speed of flow 600 yards per hour	Speed of flow 1300 yards per hour
Depth 4–5 ft.	Depth 2–3 ft.
Polluted during the winter by effluents from beet-sugar factory	Unpolluted
Bottom—mud containing decomposing organic matter	Bottom—clean stones
Higher green plants very small and young: *Sparganium simplex* and *Potamogeton lucens* dominant	Higher green plants in some quantity and of continuous growth: *Callitriche stagnalis* and *Ranunculus penicillatus* dominant

Since the River Itchen is the more rapid and shallow stream, it would be expected that the greater agitation would keep the oxygen content closer to the saturation value than in the slower and deeper River Lark.

The spring maximum of diatoms in the River Lark is considerably greater than in the River Itchen (possibly due to the recent pollution). This difference in the quantity of diatoms causes increased photosynthetic production of oxygen resulting in the higher values of supersaturation obtained on the River Lark.

The oxidation of the organic matter in the mud of the River Lark would tend to give lower minimum oxygen values than in the Itchen, where mud is absent, and this is borne out by the minimum values obtained.

The larger quantity of higher green plants in the Itchen would tend to increase the maximum oxygen values, but, at the time of the experiment, diatom activity overshadows that of the larger plants.

Summary.

Day

Photosynthesis by plants causes assimilation of carbon dioxide and liberation of oxygen, while decay of organic matter produces ammonia which is removed by the high concentration of oxygen, resulting in:

Oxygen maximum.
Ammoniacal nitrogen minimum.
p_H *value maximum.*

Night

Photosynthetic production of oxygen ceases, and the ammonia produced by organic decay is not removed. Animal and plant respiration continues, taking up oxygen and producing carbon dioxide, resulting in:

Oxygen minimum.
Ammoniacal nitrogen maximum.
p_H *value minimum.*

In conclusion, the authors wish to express their thanks to the Ministry of Agriculture and Fisheries and to Sir R. Robertson, the Government Chemist, for permission to publish these results and to Mr A. C. Gardiner, of the Ministry of Agriculture and Fisheries for his assistance in the Itchen experiment.

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Note added August 16th. Since writing the above we have seen a reference to a paper by B. Seydel "Ueber die Schwankungen des Sauerstoffgehaltes in Teichen" (*Mitt. d. Fisch-Ver. f. d. Prov. Brandenburg.* Bd. **4**, 1912–13) in which are given figures for the diurnal variation of the oxygen content of ponds. His work seems merely to amplify that of Morren [1841].

CXXVIII. BLOOD-FAT AND EXERCISE.

By JAMES WATERS THORNTON PATTERSON.

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(*Received June 19th, 1927.*)

In the course of a series of investigations into the variations of the fasting blood-fat level on diets of low and high fat contents a very definite increase after exercise was noticed. This was accidentally discovered in samples withdrawn after a game of hockey, and was quite distinct from the original line of research, but so definite as to warrant a separate investigation.

Experimental.

The method of estimation of the blood-fat content followed in this paper is that of Stewart and White [1925] and has been found eminently satisfactory.

In spite of the lengthy series of manipulations involved it was found possible to estimate the fat in a solution of known concentration within 3 %, while duplicate samples of blood gave results differing by no more than 2 %.

In addition to the warnings given by Stewart and White, the author has found it necessary to redistil all the anhydrous spirit used with sodium hydroxide, as the slight acidity of the spirit supplied was sufficient to interfere with the accuracy of results. He has, furthermore, found it necessary to interpose a CO_2 trap between the siphon system and the capillary distributing the air current to the titrating fluids. This trap consists of a concentrated solution of sodium hydroxide through which the air bubbles, having passed through the siphon system. To rely entirely on a soda-lime tower guarding the inlet of the siphon system was unsatisfactory, and, moreover, was not effective when interchange of siphon bottles had to take place. By placing this trap beyond the siphon system, however, one can interchange the bottles without any fear of the air supply becoming contaminated with CO_2.

The blood-sugar was estimated by Benedict's method [1918]. The results are all high and in later investigations the method of Folin and Wu [1920] has been used. In a series of estimations comparing the results of estimation by both these methods, the Benedict technique has been observed to give results consistently 20 % above those by the Folin-Wu method.

The cholesterol content was determined by Bloor's modification [1915] of the Autenrieth-Funk process [1913].

The air samples for the determination of the respiratory quotient were collected by means of the Douglas Bag [1911] with subsequent analysis by means of Haldane's [1918] gas analysis apparatus.

The percentage of the corpuscles was estimated by the haematocrit.

Results.

Preliminary experiments (Table I) showed that the fasting fat content remained remarkably constant for a given individual over a considerable period of time.

Table I.

Subject	Date	Fasting level mg. per 100 cc.	% variation
E. M. F.	Nov. 17, 1926 Feb. 16, 1927	661 615	6·5
I. H. M.	Dec. 4, 1926 Feb. 28, 1927	535 520	3·0
A. J.	Nov. 27, 1926 Mar. 2, 1927	696 690	1·0
W. M. A.	Dec. 3, 1926 Feb. 14, 1927	612 627	3·0
M. C. M.	Nov. 12, 1926 Feb. 10, 1927	439 433	1·5

The results of the effect of exercise are shown in Table II.

In each individual case the same amount of exercise has been performed (1) in the fasting state, (2) after the administration of 100 g. glucose by the mouth. In all cases specimen *A* is a basal fasting sample. In the experiments where glucose has been given the 100 g. are taken immediately after the collection of specimen *A*, and specimens *B* and *C* are collected at intervals of $\frac{1}{2}$ and 1 hour respectively, the patient resting in the interval. Specimen *D* is in all cases the sample immediately after exercise. Specimens *E* and *F* are collected at the times noted with the patient resting after exercise.

The subjects of the experiments were all healthy medical students.

Case E. M. F. Nov. 17, 1926.

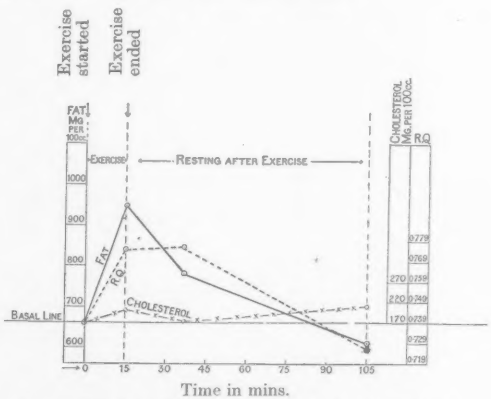

Fig. 1. Showing effect of exercise in fasting individual.

Figs. 1 and 2 represent in graphic form each type of change.

Fig. 1 (case E. M. F.) shows the effect of exercise in the fasting individual. Cholesterol and R.Q. changes are recorded.

Table II.

Case	Specimen	Remarks	Time	Fat (mg. per 100 cc.)	Sugar (mg. per 100 cc.)	Cholesterol (mg. per 100 cc.)	% corpuscles in blood	R.Q.	Work done
(1)* E. M. F.	*A*	Fasting	9.35 a.m.	661·5	—	169·4	—	0·739	Running along definite route for 15 minutes
	D	After exercise	9.55 ,,	947·2	—	202·0	—	0·775	
	E	Resting	10.15 ,,	779·9	—	173·9	—	0·776	
	F	Resting	11.45 ,,	609·7	—	212·7	—	0·726	
(2)†	*A*	Fasting	10.10 a.m.	615·6	119·0	—	38	—	Running along same route for same time
	B	½ hour after sugar	10.40 ,,	627·5	154·0	—	—	—	
	C	1 hour after sugar	11.10 ,,	577·2	129·0	—	32	—	
	D	After exercise	11.30 ,,	571·2	285·0	—	52	—	
	E	Resting	12.5 p.m.	461·7	222·0	—	—	—	
(1)* I. H. M.	*A*	Fasting	10.30 a.m.	520·9	160·0	—	34	—	Bicycle ergometer. Work done = 28,632 kg. metres
	D	After exercise	11.0 ,,	701·5	270·0	—	38	—	
	E	Resting	11.5 ,,	734·0	116·0	—	57	—	
	F	Resting	11.30 ,,	686·7	163·0	—	46	—	
(2)†	*A*	Fasting	10.15 a.m.	535·7	—	200·0	—	—	Bicycle ergometer. Repetition of above exercise. Work done = 28,632 kg. metres
	B	½ hour after sugar	—	—	—	—	—	—	
	C	1 hour after sugar	11.15 ,,	515·0	—	215·0	—	—	
	D	After exercise	11.40 ,,	467·6	—	217·0	—	—	
	E	Resting	12.20 p.m.	518·0	—	208·0	—	—	
(1)* A. J.	*A*	Fasting	10.45 a.m.	695·6	138·0	—	40	—	Bicycle ergometer. Work done = 37,794 kg. metres
	D	After exercise	11.0 ,,	843·6	235·0	—	39·6	—	
	E	Resting	11.25 ,,	858·4	253·0	—	40	—	
	F	Resting	11.35 ,,	666·0	202·0	—	38	—	
(2)†	*A*	Fasting	10.10 a.m.	689·6	126·0	208·3	—	—	Bicycle ergometer. Repetition of above exercise. Work done = 37,794 kg. metres
	B	½ hour after sugar	—	—	—	—	—	—	
	C	1 hour after sugar	11.10 ,,	680·8	235·0	204·0	—	—	
	D	After exercise	11.25 ,,	710·4	135·0	204·0	—	—	
	E	Resting	12.15 ,,	666·0	154·0	212·7	—	—	
(1)* W. M. A.	*A*	Fasting	10.18 a.m.	627·5	160·0	—	—	—	Bicycle ergometer. Work done = 57,266 kg. metres
	D	After exercise	10.40 ,,	864·3	227·0	—	—	—	
	E	Resting	11.10 ,,	488·4	175·4	—	—	—	
	F	Resting	11.30 ,,	370·0	196·0	—	—	—	
(2)†	*A*	Fasting	9.50 a.m.	612·7	—	198·0	—	—	Bicycle ergometer. Repetition of above exercise. Work done = 57,266 kg. metres
	B	½ hour after sugar	—	—	—	—	—	—	
	C	1 hour after sugar	10.50 ,,	603·8	—	190·4	—	—	
	D	After exercise	11.20 ,,	580·6	—	202·0	—	—	
	E	Resting	—	—	—	—	—	—	
(1)* M. C. M.	*A*	Fasting	9.20 a.m.	439·5	—	210·4	—	0·763	Running along definite route for 15 minutes
	D	After exercise	9.35 ,,	597·9	—	197·2	—	0·798	
	E	Resting	10.5 ,,	630·4	—	206·9	—	0·787	
	F	Resting	10.40 ,,	598·8	—	200·0	—	—	
(2)†	*A*	Fasting	10.13 a.m.	433·6	120·0	—	53	—	Running along same route for same time
	B	½ hour after sugar	10.43 ,,	245·6	206·2	—	46	—	
	C	1 hour after sugar	11.13 ,,	553·5	176·9	—	54	—	
	D	After exercise	11.32 ,,	429·2	281·7	—	—	—	
	E	Resting	11.57 ,,	432·1	217·3	—	42	—	

* Without glucose. † With glucose.

Fig. 2 (case M. C. M.) shows the effect of exercise after the ingestion of 100 g. of glucose. The relation of the sugar and fat curves during the first hour of glucose absorption (the subject resting during this period) is discussed later.

Discussion.

The most striking feature of these experiments is the constant increase in the blood-fat content after exercise in the fasting individual, an increase which does not occur after the ingestion of glucose (Figs. 1 and 2).

Case M. C. M. Feb. 10, 1927.

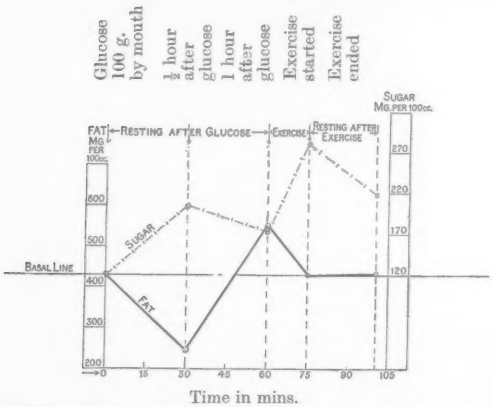

Fig. 2. Showing effect of exercise after glucose (100 g).

The increase obtained is well beyond the margin of experimental error. Thus in case E. M. F. the blood-fat content is increased by 43 %, in I. H. M. by 35 %, in A. J. by 21 %, in W. M. A. by 37 %, and in M. C. M. by 36 %.

It may be argued that this increase is due entirely to the concentration of the blood consequent upon the severe exercise, but in cases E. M. F. and M. C. M., where this question was investigated, the increase in concentration of the blood corpuscles was found to be 4 % in the former and nil in the latter, while the increase in fat content amounted to 35 % and 21 % respectively. Similar findings have resulted from the investigation of this point in cases not yet published, so that one is in a position to state that the registered increase of blood-fat after exercise is greater than that which would result from simple concentration of the blood after exercise and is, in fact, a real and not an apparent increase.

Having established the fact of a definite increase in blood-fat after exercise, one or two other points require to be settled before discussing its significance.

In the first place Leathes and Raper [1925] do not regard the variations of blood-fat content in samples of blood taken "from any accessible vein" as being of any value whatsoever in determining the nature and mechanism of fat transport and utilisation. The significance of such data derived from examination of the blood is discussed in a paper by Lyon [1923] dealing with some factors which influence dosage. Secondly, before a variation in blood-fat content can be of any importance a constant must be established. If we have no constant the increase in blood-fat after exercise loses much of its significance, since such increase might be due to normal fluctuation of the blood-fat level.

The constancy of the blood-fat content in fasting individuals.

Most observers hitherto have found extreme variations in the blood-fat fasting content in successive examinations and White [1926] has mentioned the possible factors influencing the blood-fat level and "the difficulty of obtaining any definite correlation between them."

Terroine, on the other hand [1914], working with dogs, found that any one dog would show a similar amount of fat in the blood provided the conditions remained similar (the amount being subject to a variation of 15 % either way). He found considerable variation, however, in comparing the fat contents of different dogs under similar conditions. In other words, subject to a variation of 15 %, the individual fasting fat content remained steady under similar conditions. This latter view is borne out by the results of this series of experiments on men, save that here the percentage variation is much less. Thus it will be noted (Table I) that while the range of fasting blood-fat content in the five cases of normal students is from 439 mg. to 695 mg. per 100 cc., the fasting blood-fat level in the individual shows a definite tendency to remain constant, and that the increase of blood-fat after exercise is a real and not an apparent increase.

From Table II it is evident that a very definite increase in the blood-fat content occurs after exercise in the fasting individual. It is realised that the exercise involved in a patient moving from one room to another is sufficient to upset the estimations of basal metabolism and blood-pressure for an hour afterwards. It would appear, then, that the variations in fasting blood-fat content found hitherto by other observers may be accounted for by the fact that insufficient attention had been paid to the importance of the effect of exercise as a disturbing factor.

No basal sample was withdrawn in any of the present series of cases until the patient had rested on a couch for an hour.

The increase of the blood-fat content after exercise.

This increase may be due to either of two factors. It may be a definite response to a demand for more fat on the part of the working cells, or it may be due to a non-removal of the fat from the blood-stream, the working cells either refusing it or being unable to utilise it.

The first hypothesis appears to be the more logical.

This increase in blood-fat content after exercise has been remarked upon by Gage and Fish [1924], who determined the number of fat particles in the blood by microscopic dark-field, high-power investigation. While the method seems somewhat crude, these observers noted a definite increase in fat particles, or "chylomicrons" as they call them, after exercise, and they interpreted the results as meaning that in the ordinary activity of a fasting man the fat is poured out into the blood from the depôts only as it is needed and at the rate it is needed.

They found that it took some time for the fat content to resume normal proportions, and liken the effect of exercise to that of absorption after a fatty meal where the return of the fat to its previous level is slow, the fall being due to "metabolism or assimilation or both as in the curve of digestion."

While this point of the slow return of the blood-fat content to fasting level was not investigated in this series of experiments, the indications are not in support of this finding, for in no case was the examination maintained beyond 2 hours after the exercise, and yet in three of the five cases the blood-fat content had already returned to, or gone lower than, its previous fasting level.

The variations of the blood-fat content after exercise when 100 *g. glucose had been taken prior to the exertion.*

The increase in fat content does not take place if the patient has had glucose before the exercise, despite the fact that the amount and the severity of the exercise were the same in both the investigations with glucose and without it. In all cases the fat levels after exercise when glucose has been previously taken remain practically constant (Fig. 2).

This suggests that the working cells only call upon the fat when they have insufficient carbohydrate readily available.

The respiratory quotient in relation to exercise.

The existing views on this question are somewhat conflicting and not only do the interpretations of experimental data differ, but the actual results of similar experiments vary greatly.

Some observers [see Chauveau, 1896] maintain that carbohydrate alone furnishes the energy of muscular work and that fat can only be utilised by first undergoing conversion into carbohydrate.

Others [Zuntz, 1896; Loewy, 1908; Katzenstein, 1891] find no difference in R.Q. before and during muscular work, or [Benedict and Cathcart, 1914] maintain that this is true only for light work, and that during heavy muscular work the R.Q. is slightly raised.

Another theory [Atwater and Benedict, 1903] is that the working cells utilise fat and carbohydrate in the proportions in which they are presented to them, and that if fat is to be used in the production of muscular energy, then it is oxidised directly and does not undergo a preliminary conversion into sugar.

More recently Krogh and Lindhard [1920, 1, 2] suggest that during rest and during work the proportion of fat and carbohydrate catabolised is a function of the available supplies of these substances, and that carbohydrate is formed from fat and stored when the R.Q. is below 0·8, while a corresponding transformation of carbohydrate to fat takes place when the R.Q. is above 0·9.

Benedict and Cathcart [1914] find that the R.Q. rises during prolonged and heavy work, but when the work is finished the R.Q. falls below that observed previous to the performance of the work.

The investigations into this question in the present series of cases are not sufficiently extensive to warrant any sweeping deductions. In the two cases in which the R.Q. was followed up (cases E. M. F. and M. C. M.) it will be noticed (*a*) that the initial basal R.Q. is low, (*b*) that immediately after exercise the quotient tends to rise slightly, and (*c*) after an interval of rest the R.Q. has fallen again (in one case (Fig. 1) it has fallen below that observed previous to the performance of the exercise).

Taking into account the noted increase in blood-fat after exercise, one might interpret these changes as follows.

(i) That, on the basis of the observations of Krogh and Lindhard, in the initial fasting period the low R.Q. suggests that fat is being converted into carbohydrate and stored.

(ii) That during exercise the stored carbohydrate is utilised, thus tending to force the quotient up towards unity, which is the R.Q. for pure carbohydrate combustion. Coincidently, more fat is poured out for conversion into sugar to maintain the supply of carbohydrate, so tending to lower the R.Q. The resultant slight increase in R.Q. noted immediately after exercise is the mean of these two effects.

(NOTE. The R.Q. was not taken during exercise, but immediately after it, when, it may be assumed, the physiological mechanism at work during exercise had not had time to readjust itself to resting conditions.)

(iii) After exercise, during the resting period, the depleted carbohydrate store is restored by the transformation of fat into carbohydrate with the consequent fall in R.Q. which such interconversion entails.

The relationship between the blood-sugar and blood-fat contents.

Although our knowledge at present remains insufficient for any very definite conclusions to be reached, there are certain indications that the blood-sugar and blood-fat contents are in some way related.

The evidence for such a relationship adduced in the present investigations may be briefly summarised as follows.

(1) In case M. C. M. it is noticed that after the taking of glucose (the individual resting), while the blood-sugar rises in the first half-hour and falls in the next half-hour, the blood-fat content does exactly the reverse. This has proved to be a fairly constant factor in many cases not yet published (Fig. 2).

(2) This apparent relationship in the resting individual between the blood-sugar and blood-fat, where, during absorption of sugar, the peak of the blood-sugar curve coincides with the trough of the blood-fat curve, is not maintained during exercise.

Here all the cases of exercise without glucose show an increase of blood-sugar side by side with an increase of blood-fat immediately after exercise, while in those cases where glucose was given before the exercise was started we have no increase in blood-fat but definite rise of blood-sugar in two cases

and a definite fall in another case. These changes are quite definite even when allowance of 10 % error is made for the colorimetric method of estimating the blood-sugar content.

The relation of cholesterol with the variations of blood-fat content.

Terroine [1914] found that the ratio of fatty acid to cholesterol was almost constant during the absorption of fat.

This was not substantiated by Bloor [1915], who found the variations of cholesterol during fat absorption to be irregular, small, and sometimes barely appreciable. Other observers [Hiller, Linder, Lundsgaard and Van Slyke, 1924] have confirmed Bloor's observation.

The present conception of the behaviour of cholesterol during the absorption of fat, stated briefly, is that up to a certain point the cholesterol tends to increase, but not beyond this level, depending, perhaps, on the amount available in the intestine from food or bile. The association of cholesterol with fat does not appear to be essential either to the process of absorption or of transfer from blood to tissues.

In the present series of cases where the cholesterol content was estimated it is necessary to modify the figures on account of the colorimetric method of estimation. This means an error either way of 10 %, and worked on this basis it will become evident that there is nothing conclusive in the variations—indeed, it may be deduced that the cholesterol content is unaffected, although it would be unwise to state definitely that no change occurs.

SUMMARY.

1. Figures are presented indicating a remarkable constancy of the fasting blood-fat content for the same individual.

2. A definite increase in the blood-fat content is noted as occurring in fasting individuals immediately after severe exercise.

3. There is no increase in blood-fat after exercise in the same individuals when they have received 100 g. glucose *per os* prior to the muscular exertion.

4. The variations of the R.Q. are discussed and the suggestion made that, taken in conjunction with the observed increase in blood-fat in fasting individuals after exercise, they might indicate that fat was being mobilised for its conversion into carbohydrate.

5. Indications of a relationship between the blood-fat and blood-sugar contents are noted.

6. The investigation of the cholesterol content of the blood seems to indicate that during the period of observation it remained unaffected by the exercise and, apparently, unrelated to the definite blood-fat variations.

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CXXIX. THE DISPERSED PHASE OF THE BLOOD CORPUSCLES.

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(*Received November 3rd, 1926.*)

A. Gough in an extremely interesting paper [1924] on "The nature of the red blood corpuscle" also treats the question of the dispersed phase of the blood corpuscle. He determines the dispersed phase by computing the changes of volume of the blood corpuscles in fluids of different osmotic pressure by Hamburger's principle [1898], and he comes to the conclusion that the dispersed phase constitutes two-thirds of the volume of the blood corpuscle, while the watery phase, in which the electrolytes, etc., are found to be dissolved, only forms one-third of the blood corpuscle.

The value which Gough indicates for the dispersed phase of the blood corpuscle is even greater than that which Hamburger has found, but which, according to my investigations, must be said to be too high.

It need not be specially emphasised that the knowledge of the volume of the dispersed phase is of great interest, for not till the magnitude of the dispersed phase in the plasma and blood corpuscles be known will it be possible to discuss and explain the empirically found relations of partition which have been determined for non-electrolytes capable of permeating the membrane of the blood corpuscle. Thus, if one finds that the partition of urea and acetone between the blood corpuscles and the plasma corresponds to the proportion between the magnitude of the dispersion medium in the two systems, the most reasonable explanation will be that the two substances are only found dissolved in the dispersion medium of the two systems. On finding, however, that

$$\frac{\text{Concentration in the blood corpuscles}}{\text{Concentration in the plasma}} > < \frac{\text{Dispersion medium in the blood corpuscles}}{\text{Dispersion medium in the plasma}}$$

after equilibrium has been established, it is seen that the partition cannot simply be interpreted by referring to the difference of volume of the dispersion medium. The substance in question must also be soluble in the dispersed phase of the one system, being adsorbed to the same or complexly bound.

It is therefore of great importance for the comprehension of these relations to ascertain whether the value for the dispersed phase found by Gough [1924], or that found by Ege [1920], is correct.

Gough's findings are:

Dispersion medium 30–35 % Dispersed phase 70–65 %.

Ege finds:

Dispersion medium about 60 % Dispersed phase 40 %.

By determinations performed by Hamburger's principle, I came to the result that the changes of volume of the blood corpuscles in a cane-sugar solution varied in the manner which would be expected in accordance with osmotic theories, if the dispersed phase of the blood corpuscles was 35–45 % [Ege, 1922].

When Gough, using the same method, finds a value about twice as great, this may be partly due to the circumstance that the volume of the blood corpuscles does not keep constant in such NaCl solutions as he used, but particularly to an incomplete sedimentation of the column of blood corpuscles in those experiments where the blood corpuscles are suspended in a hypertonic salt solution, which renders sedimentation much more difficult than iso- and hypo-tonic solutions [Ege, 1923]. Gough only says that all the samples were spun in a water-centrifuge for 3 hours.

Gough does not think that this has led to a complete sedimentation of the blood corpuscles, but he considers the error which will arise from this as negligible. However, he has furnished no proof of this being actually the case, and, as will easily be seen, an incomplete sedimentation may bring about a very considerable error in his determination and computation of the volume of the dispersed phase.

If the centrifuging of the blood corpuscles is continued until the whole of the column of blood corpuscles acquires the appearance of laked blood, that is to say, until an absolute sedimentation is attained, one will find that different periods of centrifuging will be required, according as the blood corpuscles are suspended in hypo- and iso-tonic solutions in which the absolute sedimentation takes place relatively rapidly, or in hypertonic solutions, with which a prolonged centrifuging is required if the same quantitative sedimentation is to be obtained. If all the samples are centrifuged for an equally long time, the values found for the volume of the blood corpuscles may become too high, particularly in the hypertonic solutions.

Even fairly small errors may lead to very great errors on computation of the volume of the dispersed phase. Thus, supposing that a complete sedimentation is attained in experiments with an isotonic fluid as well as with a fluid whose osmotic concentration is 50 % higher, the dispersed volume will be found to be = 40 %, if the first volume be = 100 and the other = 80, as becomes evident from the following formula:

$$1{\cdot}00\,(100 - x) = 1{\cdot}50\,(80 - x),$$

$$x = 40.$$

If, however, on account of imperfect centrifugalisation, the complete sedimentation were attained in the isotonic solution only, but not in the hypertonic solution, so that there still remained, for instance, 12–13 % of fluid among

the blood corpuscles, that is to say, that the volume of blood corpuscles amounted to 90 instead of the true volume of 80; the dispersed volume would then be found to be 70 %:

$$1{\cdot}00\,(100 - x) = 1{\cdot}50\,(90 - x),$$
$$x = 70.$$

The method of determining the dispersed phase introduced by Hamburger—employed by Gough—will thus easily lead to excessively high values for the dispersed phase unless the centrifuging is guaranteed to be absolutely quantitative in all the samples.

If the centrifuging is continued until the column of blood corpuscles has acquired its true volume without interstitial plasma, this manifesting itself by the column of blood taking the appearance of laked blood [Köppe, 1905] in its whole extent, a diminution of volume in the hypertonic solutions approximately corresponding to a dispersed phase of 35–45 % will be found, as has previously been mentioned.

As it might be presumed that the envelope of the blood corpuscles will resist the changes of volume, the method mentioned may, however, give rise to some objections. Another determination of the volume of the dispersed phase is derived from making solutions of non-electrolyte in equal volumes of water and blood corpuscle press-juice, and afterwards, determining the change in the freezing-point of the fluid.

8 g. of cane-sugar in water, $\Delta = 1{\cdot}22°$.

8 g. of cane-sugar in the same volume of blood corpuscle press-juice[1], $\Delta = 1{\cdot}81°$.

The volume x of the dispersed phase can then be calculated by the following formula:

$$(100 - x) \times 1{\cdot}81 = 100 \times 1{\cdot}22,$$
$$x = 33\ \%.$$

For the sake of comparison the following determination of dry matter in the blood corpuscle is mentioned:

	Dry matter	Water
Percentage of weight	34	66
Percentage of volume	28	72
	Dispersed phase	Dispersion medium
	33	67

The volume of the dispersed phase is thus 10–15 % greater than the volume found by the determination of dry matter (corresponding however rather closely to the percentage of weight).

Summary

By means of various methods of determination of the volume of the dispersed phase of the blood corpuscle, this volume is found to approach very nearly to the percentage of dry matter of the corpuscle.

[1] From this value the depression of freezing point of the ordinary corpuscle press-juice has been subtracted.

The figures given by Gough, according to which the volume of the dispersed phase is double the content of dry matter, must be due to a systematic error.

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CXXX. THE INFLUENCE OF VARIATIONS IN THE SODIUM-POTASSIUM RATIO ON THE NITROGEN AND MINERAL METABOLISM OF THE GROWING PIG. II.

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(*Received June 23rd, 1927.*)

Recent investigations on the mineral requirements of the growing animal make it increasingly evident that the adjustment of the proportions of the inorganic constituents of a ration requires as much consideration as the absolute amounts of these elements in the diet. Even though a mineral is present in a ration in sufficient amount for the animal's requirements, the presence of other minerals in unsuitable proportions may lead to insufficient assimilation and retention. The importance in animal nutrition of this balancing of the ration has been frequently emphasised by Orr [1924], who quotes various instances of the interdependence of the different mineral elements. Of special interest in this connection are sodium and potassium. The importance of the ratio of these to each other in the diet was discussed by Bunge as long ago as 1873, and since then the ratio has received more attention than the absolute requirements of each. As a matter of fact, in compounding diets the amounts of these are seldom considered. It is assumed, probably rightly, that any ordinary ration will contain sufficient.

Osborne and Mendel [1918], who carried out a series of growth experiments on rats, drew attention to the surprisingly small amounts of some of the minerals which were sufficient to permit growth. Thus less than 0·04 % of Na in the ration sufficed for normal growth, and with only 0·03 % K considerable growth was likewise attained, although the simultaneous reduction of Na and K to these minimum amounts resulted in nutritive failure. Similarly St John [1923] has found that 0·03 % of K in a wheat ration seemed to furnish an approximate maintenance supply. On the other hand, it seems probable that while these small amounts may be sufficient to cover the minimum requirements of the animal, larger amounts (especially of Na) may be necessary for the purposes of optimum growth and well-being. Thus Olson and St John [1925] have found by varying the amounts of Na in a wheat ration that the most satisfactory results, when both growth and reproduction were considered, were obtained with 0·53 % of Na.

THE Na : K RATIO OF THE RATION.

Though the absolute amounts required are so small, the ratio is of practical importance. The high proportion of K as compared with Na in the most commonly used feeding-stuffs introduces two possible dangers, viz. (1) a Na deficiency may arise not because the small Na requirement of the animal is insufficiently covered by the ration, but because the excessive proportion of K prevents the full utilisation by the animal of the Na actually present, or (2) the excessive proportion of K to Na may cause insufficient assimilation and retention of other elements.

(1) *Effect on* NaCl *content of organism. Bunge's theory.*

The whole question of the sodium-potassium ratio is intimately bound up with the theory of Bunge [1873], which for over 50 years has dominated any reference to the influence of Na and K in nutrition. Bunge, on the ground of very limited experimental data, maintained that excessive intake of potassium salts impoverishes the organism in sodium chloride. He found, in one-day experiments on himself, in which he investigated urinary excretions only, that large doses of K salts caused a large increase in the excretion of Na and Cl, this excessive loss of Na and Cl being however counterbalanced by abnormally low excretion of these elements on the three days following the K intake. Conversely, he found that a dose of sodium citrate caused increased urinary excretion of K with a slight diminution in Cl excretion. Although Bunge himself recognised the incompleteness of his experimental work, pointing out the necessity both for analysing intestinal excretions and for ascertaining the effect of continued high intake of K, he proceeded on this slender foundation to build up and elaborate the theory that has held its ground for half a century. The tendency during this time has been to overlook entirely the limitations of Bunge's experiments, and to regard as a fully demonstrated fact the theory of sodium chloride impoverishment brought about by excess of K salts. Apart from the fact that his data were insufficient to justify the theory even with regard to adult human beings, on whom the experiments were carried out, the hypothesis has been applied indiscriminately both by Bunge and others, and has been generally accepted as true for animals of any species and any age, regardless of the fact that no experimental data whatever were brought forward by him to justify the application of the theory to the young growing animal.

(*a*) *Confirmatory evidence.* Occasionally isolated results have been recorded which appear to support his theory, *e.g.* Luithlen [1912], in feeding a rabbit on green food, obtained a negative balance for Na, along with a retention of Ca, Mg and K. This he regards as an explanation of the herbivores' necessity for sodium chloride, and as a support for Bunge's theory, but the data he presents are too meagre to justify any such general conclusion. Similarly Gérard [1912], working on dogs and mice, believed that he had

obtained evidence in support of the theory of the de-sodifying action due to K salts. But the balance experiments reported by him are very short and inconclusive, while his analysis of the organs of the animals is equally inconclusive, in the absence of reliable figures for the normal content of the organs in Na and K. Again, Blum, Aubel and Hausknecht [1921], working on a nephritic patient, found that administration of K salts on a Na-poor diet caused increased excretion of Na, coupled with diuresis. The converse part of Bunge's theory, viz. increased K elimination by intake of Na salts, has also received occasional confirmation, *e.g.* Oehme [1925] states that addition of sodium chloride or sodium bicarbonate to the diet increases the elimination of K, but no details are given in the paper available. Hagentoren and Kozerski, quoted by Shohl [1923], found that sodium citrate (and sodium carbonate) increased the urinary output of K and Cl, but the excessive excretion gradually diminished. Meyer and Cohn [1911], who carried out balance experiments on infants, found that sodium chloride increased the urinary elimination of Cl, Na and K, the K balance being negative. There was a gain in weight which they attributed to a retention of Na and associated water. They found that K salts as a rule, especially the bicarbonate, caused loss of weight and increased elimination of water, Na, K and Cl. These results are quoted from Whelan [1925], the original paper not being available.

(*b*) *Contradictory evidence.* A few results have also been recorded which are not in agreement with the theory, *e.g.* Harnack and Kleine [1899] found that K salts of vegetable acids increased urinary Cl only to a very insignificant extent. Similarly v. Hoesslin [1910], who carried out metabolic experiments on dogs, considered that Bunge's theory was not confirmed. Again, lengthy feeding experiments on heifers carried out by Hart and his associates [1911] offer little support for the theory. They found, in the case of different groups of animals, fed on rations of different cereals but all having free access to salt, that the amount of sodium chloride consumed bore no relationship to the K content of the ration, either when used alone or when supplemented by K salts.

(2) *Effect on general metabolism.*

(*a*) *Calcium assimilation.* In the emphasis which Bunge lays on the Na : K ratio of the diet, and on the effect which changes in this ratio have on the excretion, he takes into account only the dependence of Na and K on each other, and their effect on Cl. But the possibility must not be overlooked that whether or not Bunge's theory is justified, an excessive proportion of K to Na in a ration may have deleterious effects in other directions. This aspect of the matter also seems to have received little attention, judging by the scanty references in the literature. That an excessive K : Na ratio may have a bad effect on Ca assimilation, and thus be the cause of rickets, was suggested by Seemann [1879] and the idea was later supported by Zander [1881]. Aron [1905] also considered it probable that such a ratio interfered with the deposit

of Ca and bone formation in cattle, while Zuntz [1912] has recorded that fodders which cause rickets usually show an excessive proportion of K to Na. Adler [1906], on the ground of metabolic experiments carried out on children, considered that Aron's results were not confirmed for human beings, but interpretation of his results is difficult on account of the shortness of the periods, and of the wide variations in the rations used in the different periods. In two of the three experiments reported his results seem rather to support than to contradict Aron's theory, and in the remaining experiment he altered the acid-base ratio of the diet as well as the Na : K ratio by giving the salts as potassium carbonate and sodium chloride, thus making a change which may have had an important effect on the Ca retention.

(*b*) *Growth and general condition.* Of the general effect on growth and welfare of high K feeding, a few experiments recently recorded are conflicting in their results. Sasaki [1924], in his feeding experiments on rats, found 1·5 : 1 to be the optimum ratio for K : Na in the foodstuffs. If the excess of K over Na were greater than this, the animals could not grow perfectly. Miller [1923], however, has stated that a diet containing K and Na in the ratio of 14 : 1 had no deleterious effect on the growth of young rats. St John [1923], in his recent experiments to which we have already made reference, found that excess of K caused loss in weight and prevented reproduction. In the series of experiments carried out in conjunction with Olson [1925], in which the percentage of Na varied from 0·228 to 2·58, we are informed by one of the authors[1] that the percentage of K in the ration was 0·32. From these figures we find that the proportion of K to Na in the ration which gave the most successful results was 0·6 : 1, while a ration in which K : Na was 1·4 : 1 did not give normal growth or successful reproduction. On the other hand, increasing the amount of Na still further led to deleterious effects on growth and reproduction. This series of experiments brings out clearly the importance for growth of suitable adjustment of the Na : K ratio.

In a recently published paper, Redina [1926] attempts to apply Loeb's theory of ion-antagonism to feeding experiments on adult mice, with special reference to the ions Na, K and Ca. He finds that the proportions of Na and K in the diet have a distinct effect on the weight-curves of the animals, but as he works with an unbalanced diet, lacking in fat and carbohydrate as well as minerals, the significance of his experiments with regard to ordinary metabolic work is not easy to determine.

So far as can be ascertained no systematic attempt has been made since the time of Bunge to deal with the question of the Na : K ratio in any of these aspects until the subject was taken up by the present authors and concurrently by Miller [1923–1926], whose work will be referred to later.

In our earlier paper [1924] we recorded experiments in which we investigated the effect, on the metabolism of the growing pig, of increasing the pro-

[1] We are indebted to Mr J. L. St John for his courtesy in supplying this information.

portion of Na to K by the addition of Na salts to a cereal ration. In those experiments it was found that the addition of sodium chloride or sodium citrate led to increased assimilation and retention of N, Ca and P. It is of interest to note that during the experimental periods in which this increased retention took place, the ratios of K : Na were 1·62 : 1 and 1·53 : 1 respectively, figures which approximate closely to the ratio of 1·5 : 1 given by Sasaki as the optimum found in his growth experiments. It was found further that while the increased Na intake did lead to increased urinary excretion of K as found by Bunge, this increase was accompanied by a diminution in faecal K, with the result that the balance showed little deviation from the normal. Thus, Bunge's theory with regard to loss of K caused by increased Na intake did not hold in our experiments on growing pigs.

Present investigation.

The experiments now to be reported, which have been carried out at intervals from 1923–1926, extend this investigation by seeking to determine the effect of increasing the proportion of K to Na by the addition of potassium citrate to the ration. The investigation has thus a twofold object: (1) to determine whether Bunge's theory, in the aspect in which it is generally applied, viz. sodium chloride impoverishment by excessive K intake, is applicable in the case of the young growing pig, and (2) to find whether the increase in the K intake affects in any way the assimilation and retention of N, Ca and P.

Experimental.

Methods. The experiments were carried out on young pigs (3–4 months old) in the manner previously described. The excretions and balances were determined for N, P, Cl, Ca, Na and K.

Basal diet. As in the previous investigation, the basal diet used in the two experiments to be described consisted of maize, oatmeal, barley-meal and blood-meal, in the quantities given below, with various salt additions as shown.

Daily food intake.

Kind of food	Pig I (control) (g.)	Pig II (g.)	Pigs III and IV (g.)
Maize	500	400	300
Oatmeal	500	400	300
Barley-meal	500	400	300
Blood-meal	50	40	30
$CaCO_3$	27·5	22	7·2
Ca phosphate	18	14·4	—
$CaCl_2$ solution (about 5 % CaO)	—	—	30 cc.
NaCl solution (approx. 20 %)	25 cc.	20 cc.	—

In each experiment the Ca content of the ration was increased by addition of Ca salts. As in the previous experiments, the animals received 10 cc. olive oil daily to assist regular defaecation and in all cases they were given distilled

water *ad lib.* In Exp. 1 the ration was further supplemented by addition of calcium phosphate and sodium chloride, in order to make the composition of the ration, with respect to minerals, approximate more closely to that of sow's milk. In Exp. 2 the ration was made exactly similar in composition to that used in the previously reported sodium citrate experiment.

Average composition of the foodstuffs.

	Total N %	P_2O_5 %	Cl %	CaO %	Na_2O %	K_2O %
Maize	1·620	0·658	0·050	0·020	0·184	0·431
Oatmeal	2·130	1·074	0·061	0·087	0·125	0·528
Barley-meal	1·801	0·834	0·110	0·062	0·103	0·648
Blood-meal	13·423	0·315	1·098	0·173	0·987	0·331

The exact intakes of N, P, Cl, etc., in each experiment are stated in the tables giving the experimental data.

Arrangement of experiments. In Exp. 1, pig I was kept as a control, the diet remaining constant for 6 weeks. Pig II, after a preliminary period of 12 days, had potassium citrate added to the ration to the amount of 64 g. daily. On the addition of this large dose of potassium citrate the pig refused to eat its complete ration for 2 days, and the K salt was therefore added in smaller amounts, gradually working up to the full dose. Thus for pig II there is a transition period of 6 days at the beginning of the experimental period (days 13–18) for which the results, being somewhat uncertain, are not included in the table. After 14 days on the full amount of potassium citrate, there followed a post-period of 18 days during which the animal received only the original ration.

In Exp. 2 both pigs were used as experimental animals. After a preliminary period of 14 days for pig III and 10 days for pig IV, each pig received 15 g. potassium citrate for 1 day, and thereafter the full dose of 30 g. daily for 15 days, after which the basal ration was fed for a post-period of 12 days.

The results of the analyses are given in Tables I–IV, and in part in Figs. 1 and 2.

Discussion of results.

I. *Effect on general metabolism.* From these experiments, and from a comparison with the results previously recorded for the Na feeding experiments, it would appear that the administration of potassium citrate has had an adverse effect on the assimilation of N, P and Ca (see Fig. 1), although the response of the individual animals to the introduction of the salt varied somewhat in degree, *e.g.* pig III seemed to be slower than pig IV in showing the effect of the potassium citrate, and to recover more quickly when the salt was withdrawn. In all cases the introduction of potassium citrate had a marked diuretic effect.

Table I. *Exp.* 1. *Pig I* (*control*).

Average daily excretions and balances in g.
Duration of experiment: 42 days.
K : Na in food intake = 2·0 : 1 throughout.

Days (inclusive)	Nitrogen					P_2O_5				
	Intake	Urine	Faeces	Total	Balance	Intake	Urine	Faeces	Total	Balance
1–7	35·05	12·55	10·09	22·64	12·41	20·23	0·25	11·87	12·12	8·11
8–14	35·05	13·17	9·19	22·36	12·69	20·23	0·27	12·28	12·55	7·68
15–21	34·80	13·21	9·21	22·42	12·38	20·23	0·22	12·35	12·57	7·66
22–28	34·48	13·81	8·18	21·99	12·49	20·25	0·21	12·34	12·55	7·70
29–35	34·48	13·75	8·38	22·13	12·35	20·25	0·27	12·78	13·05	7·20
36–42	34·48	13·77	8·22	21·99	12·49	20·25	0·26	12·68	12·94	7·31
			Cl					CaO		
1–7	4·74	4·08	0·37	4·45	0·29	22·94	1·04	13·16	14·20	8·74
8–14	4·74	3·73	0·23	3·96	0·78	22·94	1·06	13·40	14·46	8·48
15–21	4·74	3·89	0·24	4·13	0·61	22·96	0·87	13·56	14·43	8·53
22–28	4·74	3·60	0·25	3·85	0·89	22·99	0·68	14·07	14·75	8·24
29–35	4·74	3·82	0·25	4·07	0·67	22·99	0·74	14·06	14·80	8·19
36–42	4·74	3·70	0·27	3·97	0·77	22·99	0·58	14·45	15·03	7·96
			Na_2O					K_2O		
1–7	4·78	2·07	1·82	3·89	0·89	8·38	4·34	1·97	6·31	2·07
8–14	4·78	1·94	1·49	3·43	1·35	8·38	4·39	1·67	6·06	2·32
15–21	4·81	1·95	1·27	3·22	1·59	8·36	4·44	1·81	6·25	2·11
22–28	4·86	1·72	1·36	3·08	1·78	8·35	4·55	1·56	6·11	2·24
29–35	4·86	1·58	1·38	2·96	1·90	8·35	4·75	1·55	6·30	2·05
36–42	4·86	1·47	1·31	2·78	2·08	8·35	4·64	1·64	6·28	2·07

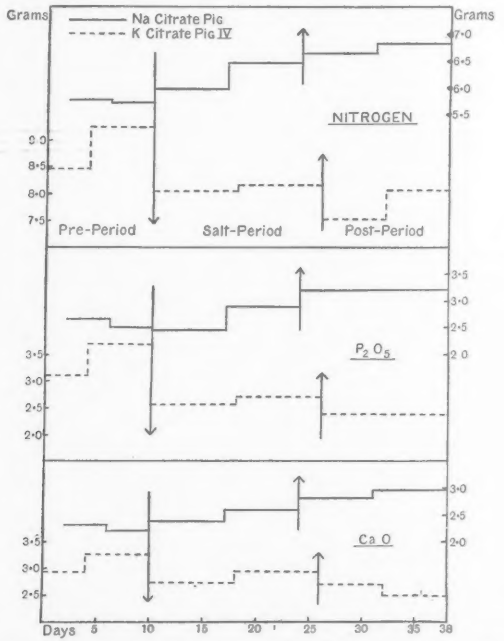

Fig. 1. Nitrogen, P_2O_5, and CaO balances for pigs receiving Na citrate and K citrate respectively, showing the opposite effects of these salts on the general metabolism.

Table II. *Exp.* 1. *Pig II.*

Average daily excretions and balances in g.
Duration of experiment: 50 days.
{K : Na in basal ration = 2·0 : 1.
{K : Na in experimental period = 10·5 : 1.

Days (inclusive)	Nitrogen					P_2O_5				
	Intake	Urine	Faeces	Total	Balance	Intake	Urine	Faeces	Total	Balance
1–6	28·04	9·78	7·97	17·75	10·29	16·18	0·20	10·96	11·16	5·02
7–12	28·04	10·36	7·93	18·29	9·75	16·18	0·23	10·72	10·95	5·23
⟶										
*19–25	28·04	11·58	7·07	18·65	9·39	16·18	0·20	11·32	11·52	4·66
26–32	28·04	11·44	7·37	18·81	9·23	16·18	0·26	11·78	12·04	4·14
⟵										
33–39	27·71	11·23	7·13	18·36	9·35	16·19	0·20	11·99	12·19	4·00
40–46	27·58	11·05	7·10	18·15	9·43	16·20	0·20	12·53	12·73	3·47
47–50	27·58	11·22	6·73	17·95	9·63	16·20	0·19	12·92	13·11	3·09
			Cl					CaO		
1–6	3·79	3·21	0·29	3·50	0·29	18·35	0·92	12·22	13·14	5·21
7–12	3·79	2·82	0·24	3·06	0·73	18·35	1·65	10·97	12·62	5·73
⟶										
19–25	3·79	2·99	0·26	3·25	0·54	18·35	0·70	13·12	13·82	4·53
26–32	3·79	3·09	0·26	3·35	0·44	18·35	0·78	13·14	13·92	4·43
33–39	3·79	2·90	0·24	3·14	0·65	18·38	1·27	12·85	14·12	4·26
⟵										
40–46	3·79	2·93	0·28	3·21	0·58	18·39	1·09	13·49	14·58	3·81
47–50	3·79	2·79	0·23	3·02	0·77	18·39	0·77	14·30	15·07	3·32
			Na_2O					K_2O		
1–6	3·82	1·88	1·80	3·68	0·14	6·70	2·81	2·19	5·00	1·70
7–12	3·82	1·95	1·67	3·62	0·20	6·70	2·99	1·89	4·88	1·82
⟶										
19–25	3·82	0·90	1·67	2·57	1·25	35·75	29·53	3·34	32·87	2·88
26–32	3·82	0·73	1·17	1·90	1·92	35·75	29·65	3·54	33·19	2·56
⟵										
33–39	3·87	1·96	1·20	3·16	0·71	6·68	3·27	2·10	5·37	1·31
40–46	3·88	2·08	1·52	3·60	0·28	6·68	2·92	2·16	5·08	1·60
47–50	3·88	1·62	1·53	3·15	0·73	6·68	2·97	2·08	5·05	1·63

⟶ Indicates the introduction of potassium citrate.
⟵ Indicates the withdrawal of potassium citrate.
* Days 13–18 omitted. See text.

Nitrogen. In all three pigs the potassium citrate feeding resulted in increased urinary excretion of N, and this increase accounted almost entirely for the diminished N balance. Only in the case of pig IV was the adverse effect on the balance partially due to increase in the faecal N. It is well known [cf. Orr, 1914] that the diuresis consequent on excessive water ingestion leads to increased urinary excretion of N, hence in the present experiments it is probable that the increase in urinary N may be ascribed to the diuresis brought about by the K feeding. This is borne out by the fact that for pig III, which seemed to be less susceptible in general than pig IV to the K salt, it was found that the diuretic effect was also less marked. It is possible, therefore, that the use of natural feeding stuffs containing a high proportion of K, if they caused no diuresis, might have little effect on the N retention.

Phosphorus. With regard to P excretion, in pigs II and IV, in spite of the diuresis, the K feeding had practically no effect on the urinary P, the diminution in balance being due to increased faecal excretion. On the other hand, pig III showed a slight decrease at first in faecal P, and the diminished balance in the second week of the K feeding was due to an increase in urinary P.

Table III. *Exp.* 2. *Pig III.*

Average daily excretions and balances in g.
Duration of experiment: 42 days.
{K : Na in basal ration = 3·0 : 1.
{K : Na in experimental period = 11·6 : 1.

Days (inclusive)	Nitrogen					P_2O_5				
	Intake	Urine	Faeces	Total	Balance	Intake	Urine	Faeces	Total	Balance
1–6	20·41	7·73	4·72	12·45	7·96	8·31	0·15	5·87	6·02	2·29
7–14	20·41	7·22	4·60	11·82	8·59	8·31	0·22	5·78	6·00	2·31
⟶										
15–22	20·41	7·57	4·44	12·01	8·40	8·31	0·31	5·51	5·82	2·49
23–30	20·41	8·38	4·31	12·69	7·72	8·31	0·58	5·75	6·33	1·98
⟵										
31–36	20·41	8·03	4·12	12·15	8·26	8·31	0·40	5·73	6·13	2·18
37–42	20·41	8·31	4·05	12·36	8·05	8·31	0·55	6·02	6·57	1·74
			Cl					CaO		
1–6	2·97	2·62	0·09	2·71	0·26	6·00	0·29	3·53	3·82	2·18
7–14	2·97	2·52	0·12	2·64	0·33	6·00	0·24	3·41	3·65	2·35
⟶										
15–22	2·97	2·52	0·14	2·66	0·31	6·00	0·18	3·18	3·36	2·64
23–30	2·97	2·55	0·16	2·71	0·26	6·00	0·20	3·66	3·86	2·14
⟵										
31–36	2·97	2·56	0·13	2·69	0·28	6·00	0·19	3·67	3·86	2·14
37–42	2·97	2·46	0·13	2·59	0·38	6·00	0·23	4·08	4·31	1·69
			Na_2O					K_2O		
1–6	1·79	0·57	0·52	1·09	0·70	4·81	1·19	2·71	3·90	0·91
7–14	1·79	0·63	0·43	1·06	0·73	4·81	1·29	2·50	3·79	1·02
⟶										
15–22	1·79	0·22	0·78	1·00	0·79	17·63	12·21	3·32	15·53	2·10
23–30	1·79	0·14	0·61	0·75	1·04	18·49	13·88	2·96	16·84	1·65
⟵										
31–36	1·79	0·48	0·53	1·01	0·78	4·81	1·89	2·23	4·12	0·69
37–42	1·79	0·40	0·22	0·62	1·17	4·81	1·40	2·56	3·96	0·85

⟶ Indicates introduction of potassium citrate.
⟵ Indicates withdrawal of potassium citrate.

Calcium. The Ca results agree for all three pigs. In every case, following an increased K intake, there was an increase in the faecal excretion of Ca, accompanied by a diminution in urinary Ca, resulting in a diminished retention. This is exactly the converse of the results obtained in the sodium citrate experiment. In that case the increased Ca balance was accompanied by increased urinary excretion, the amount of urinary Ca increasing progressively during the whole 14 day period of the sodium citrate feeding, a result which seemed to point clearly to increased assimilation of Ca. The figures now reported therefore, taken in conjunction with those of the sodium citrate experiment, seem to indicate that the Na : K ratio of a diet may have a very considerable influence on the Ca assimilation, and they form an interesting justification of the suggestions put forward by Seemann, Zander, Aron and Zuntz, to which reference has already been made.

Chlorine. The present experiments show, if we consider the two-day results, that there is a very distinct effect of the increased K intake on the urinary Cl excretion. The increase of K intake causes increased urinary excretion of Cl and a negative Cl balance for the first two-day period after the intake. This increase is, however, only transitory, being followed by a decreased excretion for the next 2 days, and, if we consider the average figures for the

Table IV. *Exp.* 2. *Pig IV.*

Average daily excretions and balances in g.
Duration of experiment: 38 days.
{K : Na in basal ration = 3·0 : 1.
{K : Na in experimental period = 11·6 : 1.

Days (inclusive)	Nitrogen					P_2O_5				
	Intake	Urine	Faeces	Total	Balance	Intake	Urine	Faeces	Total	Balance
1–4	20·41	7·23	4·71	11·94	8·47	8·31	0·25	4·96	5·21	3·10
5–10	20·41	7·00	4·15	11·15	9·26	8·31	0·18	4·45	4·63	3·68
⟶										
11–18	20·41	7·42	4·95	12·37	8·04	8·31	0·17	5·59	5·76	2·55
19–26	20·41	7·51	4·76	12·27	8·14	8·31	0·30	5·32	5·62	2·69
⟵										
27–32	20·41	8·59	4·31	12·90	7·51	8·31	0·19	5·75	5·94	2·37
33–38	20·41	8·02	4·32	12·34	8·07	8·31	0·28	5·66	5·94	2·37
			Cl					CaO		
1–4	2·97	2·19	0·21	2·40	0·57	6·00	0·28	2·79	3·07	2·93
5–10	2·97	2·41	0·22	2·63	0·34	6·00	0·29	2·46	2·75	3·25
⟶										
11–18	2·97	2·51	0·13	2·64	0·33	6·00	0·14	3·13	3·27	2·73
19–26	2·97	2·55	0·14	2·69	0·28	6·00	0·15	2·92	3·07	2·93
⟵										
27–32	2·97	2·62	0·09	2·71	0·26	6·00	0·17	3·14	3·31	2·69
33–38	2·97	2·51	0·14	2·65	0·32	6·00	0·19	3·32	3·51	2·49
			Na_2O					K_2O		
1–4	1·79	0·62	0·48	1·10	0·69	4·81	1·31	2·16	3·47	1·34
5–10	1·79	0·69	0·40	1·09	0·70	4·81	1·09	2·19	3·28	1·53
⟶										
11–18	1·79	0·34	0·99	1·33	0·46	17·63	12·27	3·08	15·35	2·28
19–26	1·79	0·30	0·96	1·26	0·53	18·49	13·23	2·99	16·22	2·27
⟵										
27–32	1·79	0·67	0·65	1·32	0·47	4·81	1·78	2·26	4·04	0·77
33–38	1·79	0·58	0·19	0·77	1·02	4·81	1·48	2·30	3·78	1·03

⟶ Indicates the introduction of potassium citrate.
⟵ Indicates the withdrawal of potassium citrate.

periods, it is found that continued excessive intake of K salts has little effect on excretions and balances. When the K salt is withdrawn there is a reverse transitory effect. Urinary Cl diminishes temporarily, but the organism adjusts itself at once. This transitory influence of increased K intake on urinary Cl is perhaps the most decided effect observed in all the potassium citrate experiments, although again the response of the animals to the change in diet varies in degree. Thus pig III showed a less decided effect when the K salt was withdrawn than pigs II and IV. In pig II the effects are exaggerated by the large dose of K salt. Table V and Fig. 2 show this effect of increased K intake on the urinary excretion of Cl, the figures being two-day excretions.

A comparison of these figures with those of the sodium citrate experiment shows that sodium citrate has the reverse effect on urinary Cl excretion, a point which was not brought out in the previous paper. Table VI and Fig. 2 give these results for the excretions and balances of Cl[1], using the corrected intake.

[1] A correction has to be made in the Cl balance figures as previously published for the Na experiments. More recent work by two of us [Husband and Godden, 1927] on the method of determining Cl in foodstuffs has shown that the method then employed gave results considerably too low in the case of cereal foods. Making this correction for the intake of Cl it will be found that the pigs instead of showing a steady negative balance were really on a small positive balance.

Table V.

	Pig II		Pig III		Pig IV	
	Urinary Cl	Cl balance	Urinary Cl	Cl balance	Urinary Cl	Cl balance
Average for last 8 days of pre-period	5·79	+1·30	5·03	+0·66	4·59	+0·90
Days 1 and 2 of potassium citrate period	9·70	Large neg. balance[1]	5·96	−0·30	5·83	−0·29
Days 3 and 4 of potassium citrate period	0·50	+6·85	4·10	+1·59	4·21	+1·49
Last 8 days of potassium citrate period	6·15	+0·92	5·10	+0·51	5·10	+0·55
Days 1 and 2 of post-period	4·15	+2·97	4·95	+0·73	4·70	+1·05
Days 3 and 4 of post-period	7·47	−0·38	5·22	+0·49	5·83	−0·10

[1] The exact figure here is uncertain, as the animal did not eat the complete ration for 2 days on account of the large dose of potassium citrate, but it may be taken as approximately −4·3.

Table VI.

	Urinary Cl	Cl balance
Last 8 days of pre-period	5·05	+0·46
Days 1 and 2 of sodium citrate period	4·29	+1·30
Days 3 and 4 of sodium citrate period	5·64	−0·17
Last 8 days of sodium citrate period	5·01	+0·51
Days 1 and 2 of post-period	5·26	+0·30
Days 3 and 4 of post-period	5·26	+0·34
Days 5 and 6 of post-period	4·96	+0·65

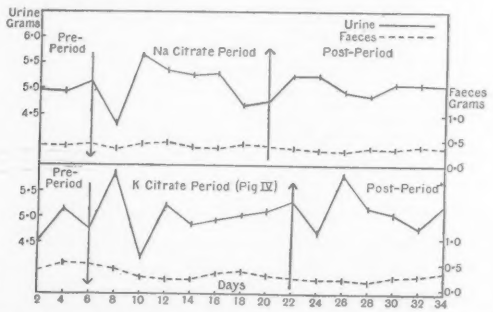

Fig. 2. Urinary and faecal excretions of chlorine for pigs receiving Na citrate and K citrate respectively, showing the opposite effects of these salts on the urinary excretion of chlorine, and the temporary nature of the disturbance.

II. *Bunge's theory of chlorine impoverishment by potassium intake.* It will be seen from a study of the above potassium citrate experiments that while the Cl results agree with those of Bunge in his experiments on himself with regard to the *temporary* increase in Cl excretion caused by increased K intake, they offer no justification of his theory that continued high intake of K may cause impoverishment of the organism in Cl. The immediate increase in urinary Cl is followed at once by decreased excretion when the K salt is withdrawn (see Tables V and VII), as Bunge himself found. We find further that this fall below normal occurs even when the high K intake is continued. From the experimental data available it seems clear that the theory of Cl impoverishment caused by high K intake is quite untenable. On the contrary, the results

obtained bring out very strikingly the rapidity with which the organism can adjust itself to sudden changes in the diet, and demonstrate clearly its ability to maintain its Cl balance in spite of large variations in the Na : K ratio of the diet.

Potassium. The increased K intake resulted in an increased K balance for all three pigs, but the excess retained during this period was got rid of in the post-period through the urine, by which also the major portion of the ingested potassium citrate was voided during the experimental period.

Sodium. While the results obtained for the Na excretions and balances in these experiments are not so consistent or so decisive in their nature as those for Cl, yet they cannot be regarded as offering any support for Bunge's theory of Na impoverishment caused by excessive K intake. In considering the results it seems advisable to point out that, owing to the method of Na estimation, the interpretation of small variations in the results is less certain than is the case with other estimations. Since the Na is estimated by difference, it follows that when large amounts of K salt are present in the urine, and consequently small amounts of material have to be taken for analysis, as is the case in the potassium citrate periods, the possibility of error in the final Na result is greatly increased. Hence only *considerable* variations in the Na figures can be accepted as forming any basis for argument.

(*a*) *Urinary sodium excretion.* Although with regard to Cl the figures we have obtained confirm Bunge's findings of a *transient* increase in urinary excretion consequent on increased K intake, we have not obtained the same confirmation as regards the excretion of Na. On only two occasions have we observed a suggestion of such increase. (1) In the transition period of Exp. 1, where the results were however somewhat vitiated by the fact that the large dose of potassium citrate caused pig II to go off its food, the urinary Na increased somewhat for the first 2 days of the period, the daily average being 1·9 g. for the last 2 days of the pre-period, and 2·2 g. for the first 2 days of the experimental period. This small difference is, however, in no way inconsistent with the two-daily variations found during the pre-period, particularly when it is remembered that the animal was not eating well. (2) Another experiment, not otherwise recorded in this paper, was conducted to throw further light on this particular point, attention being paid solely to the intake and excretion of Na and K. The urinary Na for the first day of the K period was 0·51 g. Na_2O as against an average of 0·39 g. for the 8 days of the pre-period. As the excretion for the last 2 days of the pre-period had been rather below normal, this slight increase, while it *may* be due to increased K intake, is again quite in keeping with the ordinary daily fluctuations. In this case the average urinary excretion of Na_2O for 8 days of continued high K feeding was only 0·43 g., as compared with the pre-period average of 0·39 g., and the Na balance for the period showed a slight improvement in spite of the slight increase in urinary excretion.

In no other case did we find even this amount of support of Bunge's

findings. Urinary excretion of Na diminished rather than increased during the K-feeding periods, and even when the K salt was given again for 1 day only to pigs III and IV (Table VII), at the end of the post-period recorded in Tables III and IV, no increase in urinary Na was observable, though the Cl effect already referred to was clearly shown.

Table VII.

	Pig III				Pig IV			
	Na_2O		Cl		Na_2O		Cl	
	Urine	Bal.	Urine	Bal.	Urine	Bal.	Urine	Bal.
Daily average for last 6 days of post-period	0·40	1·17	2·46	+0·38	0·58	1·02	2·51	+0·32
Potassium citrate day	0·06	1·30	3·01	−0·18	0·21	1·07	3·12	−0·24
Day after potassium citrate	0·26	1·18	1·33	+1·56	0·35	1·19	1·48	+1·41

(*b*) *Sodium balance.* As regards the Na balance also, the weight of evidence is against Bunge's theory of Na impoverishment by K feeding. In Exp. 2 the decrease in urinary Na for the K period was accompanied by increased faecal excretion of Na (cf. the results of the Na experiments already published in which increased urinary K and decreased faecal K go together). With pig III, as also with pig II, and with the animal in the short experiment already briefly mentioned, there was an improvement in the Na balance for the experimental period, while with pig IV the diminution in balance was due to the largely increased faecal excretion, which more than counterbalanced the diminished urinary excretion. Thus our results do not bear out Bunge's theory of loss of Na from the organism through increased urinary excretion. It must be borne in mind, however, that these experiments were conducted on young growing animals, while Bunge's experiments with an adult human being as subject were carried out under widely different experimental conditions.

Miller [1923–1926] has recently carried out a series of interesting experiments on "Potassium in animal nutrition," to which we have already made some reference. In his first paper, dealing with the influence of K on urinary Na and Cl excretion in pigs, Miller claimed that his results, using a starch diet, a synthetic ration, or a milk diet, agreed with those of Bunge in showing that a sudden increase of K salts in the diet caused an immediate increase in Na and Cl excretion for the following 24-hour period. He found further that the Na and Cl excretion decreased when the high K intake was continued.

From a close study of Miller's data we are unable at times to reconcile the figures in the tables with the conclusions he draws from them, particularly with regard to the effect on Na excretion. Thus in Table II, Nov. 4th, on the starch diet, there is no increase in Na excretion on giving the K salt, although the Cl figure shows the expected increase. In Table VII, on the synthetic ration, there is little or no Na increase for any of the dates on which the K salt was introduced, while in Table IX, giving the results for a pig on milk diet,

there is not only no initial increase in Na excretion, but the figures show a distinct decrease for the whole K period. It may be mentioned here that the Cl figures in Table IX agree very closely with those we have obtained both with regard to the initial rise and fall at the beginning of the K period, and also as regards the sudden drop and subsequent increase when the K salt is withdrawn.

Other instances can be found in the tables which do not fit in with Miller's general conclusions, and it seems as if Miller were ready to accept Bunge's statements in preference to the evidence of his own experimental results, instead of recognising how often the two are in conflict. We are inclined therefore to accept the results of Miller's experiments on pigs, which it must be remembered covered only urinary excretions and were not balance experiments, as offering additional support to our conclusions, instead of regarding them as a confirmation of Bunge's results. His later metabolic experiments, in which rats were used as subjects, are scarcely comparable either with his or our experiments on young pigs, or with those of Bunge on himself.

Summary.

1. The addition of potassium citrate to a ration of cereal grain fed to growing pigs, led to decreased assimilation and retention of nitrogen, phosphorus and calcium.

2. The increase of potassium salts in the ration caused an immediate increase in the urinary excretion of chlorine, followed at once by a decrease, after which continued high intake of potassium had little or no effect on the excretion. The withdrawal of the potassium salt, conversely, caused an immediate drop in urinary chlorine, followed by an increase.

3. The results obtained for growing pigs with regard to sodium excretion do not support the theory of sodium impoverishment put forward by Bunge as the result of experiments on himself.

4. For the growing animal it would appear that any adverse effect of excessive potassium content in a ration is due not to impoverishment of the organism in sodium or chlorine, but more probably to the depressing influence on the metabolism of nitrogen, phosphorus, and calcium.

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CXXXI. THE TOXICITY OF PHENYLARSINIC ACIDS FOR *BALANTIDIUM COLI* MALM. IN CULTURES, IN RELATION TO THEIR CHEMICAL CONSTITUTION.

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(*Received June 25th, 1927.*)

In the course of an examination into the effect of a variety of drugs on cultures of *Balantidium coli* it was found that out of three organic arsenic compounds used, only two showed any considerable degree of toxicity. As this seemed to indicate that a relationship might exist between toxicity and chemical constitution, the effect of a series of selected arsenicals was examined *in vitro*. The results obtained appear to be of sufficient interest to merit a brief record.

Since the discovery of the nature of atoxyl by Ehrlich in 1907, numerous papers have appeared on the use of organic arsenic compounds for protozoal diseases. Many derivatives of both ter- and quinque-valent arsenic compounds have been employed in these researches, but as yet little has been brought to light regarding the laws correlating toxicity and chemical constitution. In this connection a noteworthy feature is that different results may be obtained in toxicity tests on organisms according to whether the experiments are carried out *in vivo* or *in vitro*. Thus while sodium *p*-aminophenylarsinate has proved to be a most effective remedy for sleeping sickness, *in vitro* it is almost without action on trypanosomes.

In order to explain this discrepancy between *in vitro* and *in vivo* experiments, it has been suggested that the arsenicals undergo some change in the animal body, and that it is the resulting product and not the original substance which displays toxicity. These divergencies, however, may be more apparent than real, for toxicity tests on microscope slides may have little significance—this has been clearly shown in the case of *E. histolytica* by Dobell and Laidlaw [1926, 1]—and although trypanosomes can be readily cultivated they are in cultures so far removed from their natural conditions that little weight would attach to results from drugs tested in this way. In the present investigation this difficulty does not appear to be so marked, for it has been found possible to grow *B. coli* under conditions which seem to approximate to natural ones very closely. Particulars of this will shortly be published elsewhere.

Technique.

The strains of *B. coli* used for this work were obtained from the caecum of pigs. So far as can be determined from morphological evidence this is the same ciliate as is found in man, in which host it causes an intractable dysentery. It is possible, however, that there are really two species in the pig as described by McDonald [1922]—*B. coli* and *B. suis*—but in the material used for the toxicity tests the *B. coli* form predominated and in any case both forms are affected by drugs in exactly the same way so that we shall regard the cultures as being those of *B. coli*.

Material from the caecum of freshly killed pigs was planted in Dobell and Laidlaw's [1926, 2] medium consisting of an inspissated horse-serum slope covered with Ringer solution containing the whites of two eggs to one litre of solution. In addition a small quantity of sterile rice starch is essential. In this medium *B. coli* grows with great freedom and shows no signs of degeneration—a feature so common in the cultivation of protozoa. Indeed it seems probable that *in vitro* results on intestinal protozoa grown in this medium are much more reliable than is the case with blood-protozoa on account of the more nearly natural conditions obtained in the authors' tubes. No strain was used for experimental purposes until growth had been well maintained for several weeks.

1 % solutions of the drugs were made in distilled water and the higher dilutions, for use in the toxicity experiments, were prepared by dilution with the requisite quantity of Ringer egg-white solution. In cases where dilutions lower than 0·1 % were required, a 1 % solution was made up in the Ringer egg-white solution. In this way, the liquid medium was never diluted to any appreciable extent by addition of the drug solution. About 5–6 cc. of fluid medium containing the required drug were used in each tube and to this one drop of inoculum, obtained from a mixture of healthy 24 and 48 hour cultures was added. The tubes were incubated at 37° and examined on the second and third days after inoculation, growth in cultures always being at its best on these days. The effect of the drug was estimated by observing the numbers of living and dead organisms, the increase or decrease in these numbers, and especially by the condition of the organisms, such as the rate of ciliary movement, the size and shape of the body and the presence or absence of degenerative changes in the endoplasm. A record of life alone in cultures is not sufficient, as, in two cultures showing living organisms, one might be quite normal and the other degenerate. For this reason the tables showing our findings are annotated to show, as far as possible, the condition of the organisms in the culture tubes. Control tubes are not given in the tables in order to economise space but in all experiments at least one control tube was put up for every four drug solution tubes and usually one control for three drug solution tubes. No experiment is recorded in which the controls were unsatisfactory.

Table I. *Numbers of living* (L) *and dead* (D) *cultures on 3rd day of incubation.*

	Dilutions ...	1 : 100		1 : 250		1 : 500		1 : 750		1 : 1000		1 : 3000		1 : 5000		1 : 7500	
		L	D	L	D	L	D	L	D	L	D	L	D	L	D	L	D
A	Sodium *o*-hydroxyphenylarsinate	—	—	—	—	—	—	—	—	0	3	—	—	—	—	—	—
B	Sodium *o*-aminophenylarsinate	—	—	—	—	—	—	—	—	0	3	—	—	—	—	—	—
C	Sodium *p*-aminophenylarsinate (Atoxyl)	—	—	—	—	—	—	—	—	0	3	—	—	0	4	0	9
D	Sodium *p*-hydroxyphenylarsinate	—	—	—	—	—	—	—	—	0	7	—	—	0	3	—	—
E	Sodium *m*-aminophenylarsinate	—	—	—	—	—	—	—	—	0	8	—	—	0	5	—	—
F	Sodium 3-hydroxy-1 : 4-benzisoxazine-6-arsinate (Cyclosan)	—	—	—	—	0	5	0	5	0	14	4*	2	8	6	3	2
G	Sodium *N*-glycineamidophenylarsinate (Tryparsamide)	—	—	—	—	0	10	1	10	1	13	7*	7	10	3	7	0
H	Sodium 4-hydroxy-5-acetylaminophenylarsinate (Sodium Stovarsol)	0	5	0	4	1	6	1	7	9†	3	5	0	3	0	—	—
J	Sodium 2-hydroxy-5-acetylaminophenylarsinate (Sodium Troposan)	3‡	0	3‡	0	5	0	5	0	5	0	—	—	—	—	—	—
K	Sodium 8-acetylamino-3-hydroxy-1 : 4-benzisoxazine-6-arsinate (Sodium Parosan)	3‡	0	3	0	5	0	5	0	6	0	—	—	—	—	—	—

	Dilutions ...	1:10,000		1:15,000		1:20,000		1:30,000		1:40,000		1:50,000		1:60,000		1:70,000	
		L	D	L	D	L	D	L	D	L	D	L	D	L	D	L	D
A	Sodium *o*-hydroxyphenylarsinate	0	4	—	—	0	4	1§	3	3§	9	6	3	5	0	3	0
B	Sodium *o*-aminophenylarsinate	0	4	—	—	0	4	1§	3	0	4	5	3	5	0	4	0
C	Sodium *p*-aminophenylarsinate (Atoxyl)	4*	9	3	8	5	11	6	3	7	0	6	0	3	0	—	—
D	Sodium *p*-hydroxyphenylarsinate	0	8	0	4	5	2	7	0	4	0	4	0	—	—	—	—
E	Sodium *m*-aminophenylarsinate	0	9	3†	8	4	3	6	0	4	0	—	—	—	—	—	—
F	Sodium 3-hydroxy-1 : 4-benzisoxazine-6-arsinate (Cyclosan)	6	0	6	0	—	—	—	—	—	—	—	—	—	—	—	—
G	Sodium *N*-glycineamidophenylarsinate (Tryparsamide)	3	0	6	0	—	—	—	—	—	—	—	—	—	—	—	
H	Sodium 4-hydroxy-5-acetylaminophenylarsinate (Sodium Stovarsol)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
J	Sodium 2-hydroxy-5-acetylaminophenylarsinate (Sodium Troposan)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
K	Sodium 8-acetylamino-3-hydroxy-1 : 4-benzisoxazine-6-arsinate (Sodium Parosan)	—	—	—	—	—	—	—	—	—	—	—		—	—	—	—

* Organisms few and sluggish.
† Organisms sluggish.
‡ Slightly deformed.
§ Organisms few and degenerate.

The relation between toxicity and chemical constitution.

Of the recent work on the relation between toxicity and chemical constitution of organic arsenic compounds, researches on trypanosomes have claimed by far the greater part. In this connection reference may be made to papers by Fourneau and his collaborators [1923, 1926], King and Murch [1925], Hewitt and King [1926], and Ewins and Everett [1927]. Although it seems probable that the relation between toxicity and structure may depend to a certain extent on the specific organism experimented upon, one or two generalisations appear to be permissible from the work on trypanosomes.

(1) The aminophenylarsinic acids show considerable toxicity but the position of the amino-group is of importance, the *ortho*-isomer being most highly toxic.

(2) Marked toxicity is exhibited by the hydroxyphenylarsinic acids but the actual position of the hydroxyl grouping is less important.

(3) Fourneau found that in the treatment of trypanosomiasis, the trypanocidal power of *p*-aminophenylarsinic acid was not diminished by acetylation, but under the same treatment, the *meta*- and *ortho*-isomers showed decreased toxicity.

The compounds considered in the present investigation were examined as sodium salts and fall into three groups.

(*a*) Amino- and hydroxy-phenylarsinates: toxic to *B. coli* in dilutions above 1/20,000.

(*b*) Cyclosan, tryparsamide and stovarsol: toxic in dilutions below 1/10,000.

(*c*) Troposan and parosan: not toxic in dilutions of 1/500 and only very slightly so in 1 % dilutions.

It will be seen from our tables that the results obtained by *in vitro* work on a species of intestinal protozoa are to some extent in agreement with the generalisations given above for *in vivo* work on trypanosomes.

(1) The aminophenylarsinates show considerable toxicity, the *ortho*-isomer being most highly toxic. The position of the amino-group has a very definite effect.

(2) The substitution of a hydroxy-group in place of the amino-group does not greatly alter the toxicity, unless the degree of hydration is taken into account—see below—when the *ortho*-hydroxy salt is the most toxic examined. The position of the group seems to have a considerable effect on toxicity.

(3) Of cyclosan and tryparsamide little can be said beyond that, in the latter case, substitution in the amino-group of *p*-aminophenylarsinate has lowered the toxicity considerably.

(4) In all cases examined, addition of an acetylamino-group in a position *meta* to the arsenic grouping has resulted in a marked decrease in the parasiticidal effect. This is most marked in troposan where, by addition of the $CH_3.CO.NH$-grouping, *o*-hydroxyphenylarsinate has been rendered non-toxic.

All the toxic sodium phenylarsinates dealt with in this paper, with the exception of sodium *o*-aminophenylarsinate, are hydrated, and, when the degree of hydration is taken into account, the figures given for the lowest dilution at which the salt is not toxic are changed to those given in Table II.

Table II.

	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*
Lowest dilution at which the salt is completely non-toxic	$\frac{1}{73,500}$	$\frac{1}{60,000}$	$\frac{1}{55,000}$	$\frac{1}{41,250}$	$\frac{1}{38,800}$	$\frac{1}{11,800}$	$\frac{1}{7,727}$	$\frac{1}{3,920}$
Molecules of water	3	0	5	5	3	3	$\frac{1}{2}$	5

After making this correction certain points are clarified. In Table I *A* and *B* appear to be nearly equally toxic with a slight balance in favour of greater toxicity on the side of the amino-salt. The same is true of *D* and *E*. In Table II, on the other hand, there are well-defined intervals between the different toxic points and the *ortho*-hydroxy salt is definitely the more toxic.

The order of the toxicity of the compounds, however, still remains as given above. Previous workers do not appear to have taken the matter of hydration into account.

We wish to express our indebtedness to Dr A. J. Ewins, of Messrs May and Baker, Ltd., for a gift of most of the chemicals used in the investigation and for much useful information.

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CXXXII. THE EFFECT OF ALKALOSIS ON THE EXCRETION OF CHLORIDE AND ON CARBOHYDRATE METABOLISM.

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The phenomena accompanying alkalosis have received considerable attention in recent years, both because of their clinical bearing in such cases as tetany and because of certain peculiar consequences of theoretical interest.

Apart from its occurrence in pathological conditions alkalosis may be induced by two main methods in the normal subject: (*a*) by overbreathing, (*b*) by administration of alkalis. The former method is a severe one for the subject, as if it is persisted in for any length of time marked symptoms of tetany are produced. The expulsion of CO_2 from the alveoli leads to an uncompensated alkalosis and the p_H of the blood may change markedly as the attempt to compensate becomes futile. The attempt to compensate consists in lowering the volume of expired air and the excretion of alkali in the urine. In addition, there occurs an excretion of ketonic substances in the urine.

In inducing alkalosis by the administration of alkalis the effects produced are much milder because the compensation of the condition is much more effective. Large doses of sodium bicarbonate or acetate may be taken without any obvious disturbance other than an occasional diarrhoea and, of course, marked thirst. The compensatory mechanism again consists in diminished breathing, retention of CO_2 in the alveoli and excretion of large quantities of alkali in the urine.

No symptoms of tetany have been seen in the experiments in this paper.

Of course, in a case of lowered kidney efficiency, a flooding of the tissues with alkali without rapid excretion may bring about tetanic symptoms. Such cases are on record.

As in the case of alkalosis following overbreathing, the alkalosis following alkali administration is sometimes followed by ketosis.

The production of ketosis in alkalosis has been explained in two principal ways: (1) that the alkali in being excreted carries with it the ketonic acids from the seat of their formation, that is a so-called "washing-out" effect; (2) that it is due to a disturbance of carbohydrate metabolism. It is clear that the determination of the correct view is of the first importance in justifying or condemning alkali therapy in diabetes, or other conditions accompanied by acidosis.

In this paper the method of inducing alkalosis was by administration of alkali by mouth. The questions which are considered are the following.

(1) What is the rate of excretion of sodium bicarbonate in normal man after different doses?

(2) Under what conditions is the alkalosis accompanied by ketosis?

(3) The effect of alkalosis on ketosis in starvation.

(4) The effect of alkalosis on carbohydrate metabolism.

(5) The effect of alkalosis on the action of insulin.

(6) The effect on glycogen formation.

The methods of analysis were the following.

(1) For the determination of bicarbonate in the urine the apparatus of Van Slyke for blood-gas analysis was used. The urine was diluted so that 1 cc. gave not more than about 0·7 cc. of CO_2 on treatment with acid. The volume of CO_2 was found by direct absorption in normal NaOH, corrected and expressed as $NaHCO_3$.

(2) Urine- and blood-chlorides were determined by Van Slyke's method.

(3) Blood-sugar estimations were made by MacLean's method. Urine-sugar was estimated by Shaffer-Hartmann's or Bertrand's method.

(4) Glycogen estimations were made by Pflüger's method. The animals used were white rats. They were killed by a smart blow on the head and immediate decapitation. Violent reflex spasms occurred for a few seconds. The liver was quickly removed, dropped into a weighed wide-mouthed flask and quickly weighed to the nearest centigram. With a little practice this can be done so quickly that the loss of water vapour is negligible.

The final filtrate was always made up to 100 cc. and 25 cc. were hydrolysed for 3 hours with HCl, the glucose being estimated by Bertrand's method.

While the liver was being weighed the carcase was rapidly skinned, eviscerated, weighed, minced, and about 40 g. worked up for glycogen. The final filtrate was made up to 100 cc. and 25 or 50 cc. were hydrolysed.

(5) Phosphates in the urine were determined by Briggs' modification of the Bell-Doisy method.

(6) Respiratory quotients were determined by means of the Douglas bag and the Haldane apparatus.

(7) Alveolar CO_2 values were obtained by averaging the analyses of the inspiratory and expiratory samples.

Exp. 1. The excretion of $NaHCO_3$ in a normal man. The subject was the writer. A meal had been taken at 9.30 a.m. At 12 noon 10 g. $NaHCO_3$ in 100 cc. water were ingested.

No disturbance of any kind was experienced and there was no ketosis. The concentration of $NaHCO_3$ in the urine reached a maximum in 2 hours and remained practically constant for the following 2 hours. The total excretion of alkali in $4\frac{1}{2}$ hours was 2·58 g. and 8 hours later the urine was still strongly alkaline to litmus. The excretion of NaCl must be compared with the ordinary excretion after the morning meal.

	Urine				
		$NaHCO_3$ g.		NaCl g.	
Time	Vol. cc.	%	Total per hour	%	Total per hour
11.47 a.m.	57	—	—	1·485	—
12.00 noon	6	—	—	1·695	0·471
12.30 p.m.	24	0·135	0·065	1·594	0·766
1.00 ,,	43·5	0·842	0·732	1·083	0·942
1.30 ,,	42	0·914	0·768	0·960	0·806
2.00 ,,	42	1·021	0·858	0·906	0·762
2.30 ,,	32	1·093	0·700	0·854	0·546
3.00 ,,	26	1·111	0·578	0·765	0·398
3.30 ,,	26·5	1·111	0·589	0·765	0·404
4·30 ,,	44	1·052	0·463	0·800	0·352

Exp. 2. The excretion of chloride and inorganic phosphate after the morning meal. Breakfast 9.30 a.m. 100 cc. water at 12.20 p.m. Subject M. W. G.

	Urine				
		NaCl g.		Inorganic phosphate mg. P	
Time 11.20 a.m.–	Vol. cc.	%	Total per hour	%	Total per hour
12.20 p.m.	52	1·465	0·762	26·64	13·85
1.20 ,,	58	1·646	0·955	17·30	10·03
2.20 ,,	50	1·571	0·789	48·62	24·31
3.20 ,,	56	1·594	0·893	57·81	32·37
4.20 ,,	47	1·566	0·736	56·42	26·52
5.20 ,,	39	1·472	0·574	64·66	25·22

It is now seen that although the kidney was slowly excreting $NaHCO_3$ in Exp. 1 it was beginning to find difficulty in excreting chloride. This will be seen much more markedly in the next experiment.

Exp. 3. The excretion of $NaHCO_3$, NaCl and inorganic P after three doses of $NaHCO_3$ of 10 g. each at 11.38 a.m., 12.33 p.m., 1.35 p.m.; each dose in 40 cc. water. Breakfast at 9.30 a.m.

	Urine						
		$NaHCO_3$ g.		NaCl g.		Inorg. P mg.	
Time	Vol. cc.	%	Total per hour	%	Total per hour	%	Total per hour
11–11.30 a.m.	31	—	—	1·46	0·90	32·3	20·64
12.30 p.m.	68	0·466	0·317	1·30	0·88	12·8	8·70
1.30 ,,	53	1·606	0·851	0·56	0·30	30·9	32·75
2.30 ,,	91	1·820	1·656	0·268	0·24	34·6	31·49
3.30 ,,	117	2·998	3·508	0·218	0·26	23·4	27·38
4.30 ,,	90	2·922	2·630	0·206	0·19	26·4	23·76
5·30 ,,	54	2·656	1·434	0·150	0·08	38·7	20·90
6.30 ,,	44	2·427	1·068	—	—	56·7	24·95
7·20 ,,	22	1·898	0·500	—	—	80·0	17·6

In this experiment about 12 g. of $NaHCO_3$ were excreted in 7 hours and the urine was still alkaline at noon the following day.

There was no ketosis throughout this experiment and the compensation of the alkalosis was perfect. The marked fall in the output of chloride was very striking and has been observed in every experiment in which the kidney

was made to excrete large amounts of alkali. This fall may be variously interpreted and without entering into a discussion on the matter on this occasion we may briefly say that there seem to be two possible views.

(1) The kidney may only be capable of excreting up to a certain limiting concentration of ions, which might be calculable from a summation of the molar concentrations of each electrolyte in the urine multiplied by its ionisation coefficient. The work of Adolph [1923] in this connection is suggestive.

(2) Although we have given our chloride figures as NaCl it must be remembered that after all what is estimated is chlorine ions, *i.e.* negative ions. It may be that the retention of chlorine is a compensatory mechanism to deal with the alkalosis.

No appreciable fall in the excretion of inorganic phosphate has been found in alkalosis. Evidence has also been obtained that there is no interference with the excretion of inorganic sulphate.

The following experiment was carried out to see if any evidence of retention of chlorine in the blood could be obtained in alkalosis.

Exp. 4. Breakfast 9.15 a.m. 30 g. $NaHCO_3$ in 150 cc. water at 1.15 p.m. Blood taken from arm vein for blood- and plasma-chlorides, collected under oil.

	Urine						Blood NaCl g. %	
			$NaHCO_3$ g.		NaCl g.			
Time 10.15 a.m.–	Vol. cc.	Ke-tones	%	Total per hour	%	Total per hour	Whole blood	Plasma
1.15 p.m.	166	–	—	—	1·715	0·949	0·478	0·598
2.15 „	75	–	0·888	0·666	1·030	0·773	0·447	0·579
3.40 „	170	∓	1·844	2·213	0·399	0·479	0·471	0·603
4.55 „	164	+	2·814	3·692	0·297	0·389	—	—
5.30 „	63	+	2·407	2·600	0·220	0·238	—	—
8.15 „	217	+	3·240	2·557	0·208	0·164	—	—
9.55 „	125	∓	1·815	1·361	0·246	0·185	—	—

In the $8\frac{2}{3}$ hours of this experiment the total excretion of $NaHCO_3$ was 19·23 g., the maximum hourly excretion being 3·69 g. It will be observed that the mild ketosis appeared when the concentration of alkali in the urine was 1·84 %. This ketosis was associated with only 4–5 mg. % of acetone. This became more marked as the excretion of alkali continued but at 9.55 p.m. when the concentration of bicarbonate was 1·815 % the reaction was only just perceptible. This matter will be returned to later. The chloride excretion as before fell very markedly, but the blood chlorides, whilst indicating some initial dilution of the blood, soon returned to the original level both in whole blood and plasma.

After a dose of 30 g. of bicarbonate one very soon feels a large accumulation of fluid in the intestine. This fluid is fairly certainly rich in chloride which probably makes a large call on the store of salt in the body. The blood-chloride is, as we have seen, kept up to normal levels and it appears that the kidney is responsible for this by refusing to excrete chloride in these conditions at the same rate as it would normally.

In the next experiment a period of starvation was undergone before inducing alkalosis in order to see how far the diminution in chloride excretion could be pushed.

Exp. 5. (*a*) Chloride excretion in a normal man after 13½ hours' starvation. 100 cc. water at 11.30 a.m. Subject M. W. G.

	Urine		
		NaCl g.	
Time	Vol. cc.	%	Total per hour
9–11.30 a.m.	86	1·280	0·440
12.30 p.m.	41	1·371	0·562
1.30 „	53	1·443	0·765
2.30 „	42	1·226	0·515
3.30 „	34	1·191	0·405
4.30 „	23	0·938	0·216
5.30 „	23	0·764	0·176

(*b*) Effect of bicarbonate ingestion on chloride excretion in a normal man after 14 hours' starvation. Subject M. W. G. 25 g. $NaHCO_3$ in 100 cc. water at 12 noon.

	Urine					
			NaCl g.		$NaHCO_3$ g.	
Time	Vol. cc.	Rothera test	%	Total per hour	%	Total per hour
12.00 noon	78	–	1·006	0·392	—	—
1.00 p.m.	51	–	0·934	0·476	0·575	0·293
2.00 „	88	–	0·430	0·378	1·978	1·741
3.00 „	68	–	0·340	0·231	2·427	1·650
4.00 „	50	–	0·278	0·139	2·445	1·223
5.00 „	46	–	0·245	0·113	2·481	1·141
5.45 „	32	+	0·232	0·099	2·660	1·135

The fall in chloride excretion in (*b*) is very marked and levels are reached which ordinarily are only attained after 40 hours' starvation. In this experiment the total excretion of alkali was 7·2 g. in 6 hours and the urine was alkaline late the following day. A definite ketosis appeared 5¾ hours after the $NaHCO_3$ was taken, when there was 2·66 % in the urine. The intensity of the ketosis was such as to make it very unlikely that it was due to a "washing-out" effect. In Exp. 4 the ketosis was more intense when the urine contained 1·84 % $NaHCO_3$ than when in the above experiment the urinary alkali was 2·44 %.

In fact it seems that the more rapidly the alkali is excreted the less likelihood is there that a ketosis will appear.

Further, the value of the very small quantities of ketones excreted in these conditions is negligible as a neutralising mechanism.

In Exp. 5 (*b*) the minimum hourly output of chloride is seen to be 0·099 g. The following experiment was carried out to see if values below this could be obtained by increasing the period of starvation.

Exp. 6. (*a*) The excretion of chloride after 38 hours' starvation. Subject M. W. G. 100 cc. water at 8 a.m.

	Urine		
		NaCl g.	
Time	Vol. cc.	%	Total per hour
9.05–10.00 a.m.	24	0·412	0·108
11.10 a.m.	29	0·483	0·120
11.45 „	16	0·395	0·108
12.00 noon	7	0·412	0·116
12.30 p.m.	12	0·412	0·099
1.00 „	13	0·332	0·086
1.30 „	13	0·365	0·095
2.00 „	16	0·355	0·114
2.30 „	13	0·254	0·066
2.45 „	9	0·294	0·106
3.15 „	15	0·370	0·111

The hourly excretion of chloride is seen to be very steady during the 6 hours of the experiment, but this is not always the case. In the following table the chloride output is much higher but the dose of $NaHCO_3$ brings it down to minimal levels in about 2 hours.

(*b*) A fast of 41 hours was undergone and 20 g. of $NaHCO_3$ in 100 cc. water were taken at 11.30 a.m. Subject M. W. G.

	Urine				
		NaCl g.		$NaHCO_3$ g.	
Time	Vol. cc.	%	Total per hour	%	Total per hour
9.30 a.m.	188	0·295	0·052	—	—
11.00 „	52·5	0·781	0·273	—	—
11.30 „	14·5	0·797	0·231	—	—
12.00 noon	17	0·720	0·245	—	—
12.35 p.m.	32	0·510	0·280	0·950	0·54
1.08 „	51	0·369	0·342	1·127	1·045
1.15–1.45 p.m.	30	0·217	0·130	1·118	0·671
2.10 „	31	0·126	0·094	1·478	1·100
2.41 „	35·5	—	—	2·084	1·480
3.11 „	42	0·152	0·128	2·264	1·902
3.35 „	24	0·171	0·103	2·290	1·374

It is seen that even after 41 hours of complete fasting and when the kidney in addition is excreting as much as 1·9 g. $NaHCO_3$ per hour the output of chloride is still at the rate of about 0·1 g. per hour. This minimum is rarely diminished and it would appear that the concentration at which the alkali is excreted is limited by this minimum of chloride. Summation of the molar concentrations of the bicarbonate and chloride within certain limits gave figures of some constancy in particular experiments.

For example, the following figures were obtained in one case after 30 g. $NaHCO_3$: 0·027, 0·029, 0·033, 0·034, 0·026, 0·023, 0·024, 0·016.

These values were not corrected for ionisation. The failure in constancy occurred generally during the fall in bicarbonate excretion, the recovery in the chloride being much slower than the fall in the alkali.

Exp. 7. Seeing that the ingestion of alkali brings about a markedly diminished excretion of chloride, it was of interest to see if a similar effect could be demonstrated in the stomach.

Mr W. J. Griffiths of this laboratory, who is very expert in gastric analysis, kindly undertook to be the subject of this experiment.

The experiment was started 4½ hours after a moderate breakfast. The stomach-tube was passed and the stomach washed out until the washings were clear. 25 g. of $NaHCO_3$ were taken in 100 cc. water at 1 p.m. Gastric and urinary samples were taken about every 15 minutes.

	Gastric contents			Urine		
Time	Reaction to Congo red	% NaCl	Time 8.30 a.m.–	Vol. cc.	NaCl g. per hour	$NaHCO_3$ g. per hour
1.15 p.m.	Neg.	0·317	1.00 p.m.	316	0·621	0·020
1.30 „	Neg.	0·317	1·15 „	55	1·335	0·033
1.45 „	Neg.	0·311	1·30 „	21	0·454	0·063
2.00 „	Neg.	0·355	1·47 „	82·5	1·243	0·833
2.15 „	Neg.	0·437	2.00 „	13·5	0·298	0·703
2.34 „	Neg.	0·466	2.15 „	37	0·789	1·751
2.45 „	Neg.	0·484	2.34 „	40	0·440	1·760
3.00 „	Neg.	—	2.45 „	33	0·363	1·760
3.15 „	Neg.	0·640	3.00 „	26·5	0·389	1·698
3.30 „	Neg.	0·638	3.15 „	22	0·303	1·293
3.45 „	Acid	—	3.30 „	—	—	—
4.15 „	Acid	—	—	—	—	—

The steady rise in chloride in the stomach, accompanied by a fall in the urinary chlorides, makes it clear that whatever the mechanism for sparing chloride may be the gastric glands do not assist in its maintenance. We may further regard these values of stomach chlorides as corroborating the conclusion that the supply of chlorine from the blood is not interfered with.

The effect of alkalosis on carbohydrate metabolism and on ketosis.

It was shown by Haldane and his co-workers [1924] that alkalosis had some inhibitory effect on carbohydrate metabolism. Alkalosis, whether induced by overbreathing or alkali ingestion, lowers the normal tolerance to carbohydrate, that is to say, that doses normally producing a good rise in R.Q. and not followed by glycosuria gave rise in alkalosis to glycosuria and practically no change in the respiratory metabolism. Accompanying these phenomena, markedly abnormal blood-sugar curves were found.

The following experiments were designed to throw further light on this question.

Exp. 1. This experiment was carried out to see if the mere presence of alkali in the gut affected sugar metabolism. 5½ hours after a light meal 25 g. of $NaHCO_3$ in 100 cc. water were ingested and 14 minutes later 50 g. of glucose were taken. Subject M. W. G. Alkali at 2.46 p.m. Sugar at 3 p.m.

		Urine			
				$NaHCO_3$ g.	
Time	Blood-sugar %	Vol. cc.	Rothera test	%	Total per hour
2.20 p.m.	—	181	–	—	—
2.45 „	0·087	—	–	—	—
3.00 „	0·090	12	–	0·009	0·001
3.30 „	0·141	19	–	0·119	0·025
4.00 „	0·135	33	–	0·779	0·514
4.30 „	0·090	45	–	1·383	1·224

The blood-sugar values of this subject in analogous conditions without alkali are of the following order: 0·102, 0·150, 0·125, 0·095, 0·090 %. Thus it appears that the mere administration of alkali and an almost immediate ingestion of sugar do not give rise to an abnormal blood-sugar curve; nor was there any appearance of sugar in the urine. The situation is, however, quite different if a period of some hours is allowed to elapse between the taking of the alkali and the sugar. This was done in the next experiment.

Exp. 2. The conditions were similar to those in Exp. 1. 30 g. $NaHCO_3$ were taken at 12 noon and 50 g. glucose at 3.35 p.m. Subject M. W. G.

		Urine				
Time	Blood-sugar %	Time	Vol. cc.	Rothera test	Glucose g. %	$NaHCO_3$ g. %
12.00 noon	0·133	—	—	—	—	—
1.30 p.m.	0·115	1.32 p.m.	285	–	—	1·341
3.10 „	0·102	1.50	—	–	—	—
4.5 „	0·146	2.35	113	±	—	2·019
4.35 „	0·171	3.30	76	+	0·034	2·430
5.05 „	0·120	4.07	38	+	0·034	2·502
5.35 „	0·090	4.37	23	+	0·466	1·841
		5.10	34	+	0·140	1·573
		5.41	—	±	0·070	1·090

The blood-sugar curve here produced is clearly of the so-called lag type. There is a smart rise above the threshold and a rapid readjustment to normal levels. With larger doses of alkali the curve can be made to reach higher levels and the return to resting values can be delayed by some hours. Traces of reducing substances were present in the urine between 5.10 and 5.41 p.m. when the blood-sugar was certainly below 0·12 %. Some slight effect on the threshold value of glucose seems to have been produced but it is difficult to be sure about this.

It is to be observed that the storage of sugar was in full activity at a time when 1·84 % of $NaHCO_3$ was being excreted by the kidney. Further, throughout the whole experiment only 6·4 g. of alkali were excreted, so that, even allowing for the loss which certainly takes place by the bowel, there was still a great deal being absorbed.

It would appear then that the interference with sugar storage takes place most markedly at a time when the excretion of alkali is at a maximum. The effect can pass off rapidly if the removal of the alkali *via* the kidney is sufficiently quick to prevent any accumulation of it above a certain optimum level in the tissues. It is also seen that a definite ketosis appeared in spite of

the administered glucose. This ketosis reached a maximum when the interference with adequate sugar metabolism was most pronounced and almost disappeared as the excess alkali was thrown out.

There can be little doubt that alkalosis when well established produces a definite diminution in the power to store sugar. In the next experiment evidence is presented that there is also some interference with oxidation as indicated by respiratory quotient determinations.

Exp. 3. The effect of alkalosis in a case of markedly increased sugar tolerance. Subject M.

The case chosen was a eunuchoid whose sugar tolerance had been examined on two previous occasions. On the first occasion, 19. iii. 1926, 50 g. of glucose 12 hours after food gave rise to the following blood-sugar values at half-hourly intervals: 0·075, 0·104, 0·113, 0·091, 0·085 %. On the second occasion, 22. ii. 1927, the R.Q. values were also obtained:

Blood-sugar %	0·072	0·100	0·104	0·113	0·081
R.Q.	0·88	1·01	1·02	0·99	0·95

On 1. iii. 1927, 40 g. $NaHCO_3$ were given at 11.0 a.m. and 50 g. glucose at 12.30 p.m. The conditions were similar to those in the previous tests.

Time ...	10.30	11.30	12.00	12.30	1.00	1.15	1.30	2.00	2.30	3.00	3.30
Blood-sugar %	0·085	0·090	0·095	0·106	0·135	0·122	0·120	0·120	0·133	0·111	—
R.Q. ...	0·86	—	0·91	—	0·92	—	0·876	0·895	—	—	—

Such blood-sugar values have never been found in this patient under ordinary conditions and there can be little doubt that the storage mechanism for sugar has in some way been rendered less active than normally. Further, the rise in R.Q. was much below that which occurred in the control experiment and we must suppose that oxidation was interfered with, although by no means completely inhibited.

Exp. 4. The effect of alkalosis in a case of severe glycosuria without ketosis. Subject Mrs V.

The case chosen was a woman of 50 years weighing 15 st. 10 lb. and still gaining weight slowly. A dose of 75 g. of glucose 14 hours after food gave the following blood-sugar values at half-hourly intervals: 0·336, 0·327, 0·400, 0·376, 0·312 %, and in the 2½ hours of the experiment the urine contained 24·5 g. glucose.

This patient constantly excreted between 100 and 120 g. sugar a day even on a restricted diet. She had no ketosis at any time during 12 months' intermittent examination. It must apparently be supposed that in this type of case adequate fat metabolism is maintained by means of carbohydrate formed from either fat or protein or both, and that this is more readily utilisable than ordinary exogenous carbohydrate.

In this experiment a light breakfast was taken by the patient at 8 a.m. and a dose of 30 g. of $NaHCO_3$ was given at 11.38 a.m.

Time	Urine: Vol. cc.	Sugar g. %	Total sugar (g.)	Rothera test	NaCl g. per hour	$NaHCO_3$ g. %	R.Q.
10.55 a.m.	185	5·83	10·79	–	0·335	—	0·74
11.35 „	152	8·25	12·54	–	0·748	—	—
11.38 „	30 g. $NaHCO_3$ in 100 cc. water						
1.00 p.m.	400	4·70	18·80	+	1·028	0·523	0·70
2.07 „	282	3·30	9·31	+	0·488	1·125	0·72
3.05 „	173	3·15	5·45	+	0·230	1·079	—
4.05 „	142	—	—	+	0·145	1·033	0·70
5.00 „	78	—	—	+	0·083	0·888	—

The blood-sugar values were:

Time	11.20	12.00	2.05	3.00	4.00	4.50
Blood-sugar %	0·457	0·434	0·347	0·349	0·304	0·316

In 4½ hours 56·89 g. of sugar were excreted after only a very light meal at 8 a.m., but it is seen that even with 8·25 % of glucose in the urine there was no trace of ketonic acids. Judging from the R.Q. very little sugar was being metabolised, but even this little was thrown out of action by the alkali and a ketosis was established 1½ hours after the $NaHCO_3$ was taken. Still it must be observed that the amount of ketones excreted was not such as one would associate with a complete inability to metabolise sugar. Anti-ketogenic substances must still have been present and in action.

The total bicarbonate excreted was 9·28 g. in 5½ hours which is not less rapid than in the normal subject. It is further to be observed that the excretion of sugar was not interfered with.

As in the previous experiments there occurred a striking fall in the urinary chlorides.

The effect of alkalosis on the ketosis of starvation.

Administration of alkali to diabetics leads to an increased excretion of ketonic acids. The same thing occurs in the ketosis of starvation in normal subjects. The ketosis induced in rats by feeding on a high fat diet is much increased by alkali feeding. Lactic acid may also be increased in the urine in these conditions and it was tentatively suggested by Macleod and Knapp [1919] that these acids might be of importance in the regulation of blood reaction. As a matter of fact the quantities in which these acids are produced are so small that they can only be of very little value as a neutralising mechanism. In our view the increase in ketosis in alkalosis is an expression of an interference with carbohydrate metabolism.

The following experiment is regarded as strong evidence in favour of this view.

Exp. 5. The writer underwent a period of 41 hours' starvation after which a marked ketosis was present. It had been found on many previous occasions that such a ketosis can be completely destroyed by 50 g. glucose in about 1 hour and always in less than 1¼ hours [Goldblatt, 1925].

In this experiment 20 g. $NaHCO_3$ were taken at 11.30 a.m. and 50 g. glucose at 12.45 p.m.

Time	Blood-sugar g. %	Urine: Vol. cc.	Urine: $NaHCO_3$ g. %	Urine: Glucose g. %	Urine: Acetone mg. per hour	R.Q.	Alveolar CO_2 %	Acetone in expired air per 2 mins. mg.
9.30 a.m.	—	188	—	—	10·60	—	—	—
10.12 „	0·075	—	—	—	—	0·67	—	0·286
10.30 „	—	—	—	—	—	—	5·585	—
10.45 „	0·076	—	—	—	—	—	—	—
11.00 „	—	52·5	—	—	35·70	—	—	0·182
11.20 „	—	—	—	—	—	—	5·655	—
11.30 „	—	14·5	—	—	24·36	—	—	—
11.46 „	—	—	—	—	—	0·68	—	—
12.00 „	0·077	17	—	—	24·62	—	—	—
12.20 „	—	—	—	—	—	—	6·075	0·092
12.35 „	—	32	0·95	—	45·42	—	—	—
1.05 p.m.	0·113	—	—	—	—	0·64	—	0·068
1.08 „	—	51	1·127	—	—	—	—	—
1.17 „	0·164	—	—	—	43·40	—	—	0·077
1.22 „	0·175	—	—	—	—	—	—	—
1.45 „	0·187	30	1·118	0·061	23·40	0·74	5·983	—
2.02 „	0·209	—	—	—	—	—	—	0·120
2.10 „	—	31	1·478	0·400	—	—	—	—
2.17 „	0·168	—	—	—	—	—	—	—
2.35 „	0·115	—	—	—	—	—	—	—
3.11 „	0·078	—	2·264	—	23·00	—	—	—

The findings in this experiment may be summarised as follows.

(1) The already diminished tolerance to sugar existing after starvation is accentuated in the alkalosed starving subject.

(2) The ketosis was most markedly increased during the second hour after the alkali was taken and when the concentration of $NaHCO_3$ in the urine was only about 1 %.

(3) The ketosis had not disappeared and was only slightly diminished 9 hours after the glucose was taken. In the table only the figures up to $2\frac{1}{2}$ hours after the sugar are given, but the nitroprusside test was still strongly positive $6\frac{1}{2}$ hours after this.

(4) The excretion of ketonic substances in the breath is greatly diminished under the influence of alkali.

(5) The rise in alveolar CO_2 is brought in as a compensatory effect and reaches a maximum long before the kidney reaches its maximum excretory function. The alveolar mechanism appears to be the first line of defence until the kidney gets rid of the excess bicarbonate. This fact is shown very well in the following table of an experiment in which the writer took 30 g. $NaHCO_3$ about 3 hours after a meal.

Time	11.48	12.45	1.13	1.55	2.35	3.20	4.57	5.40
Alveolar CO_2 % ...	5·37	5·58	5·89	6·38	—	6·45	5·94	—
Urinary $NaHCO_3$ g. %	0	0·744	1·822	2·564	2·676	2·137	3·243	2·764

The evidence presented in the foregoing experiments leaves little doubt that alkalosis is accompanied by a definite diminution in carbohydrate storage and oxidation. The next logical step seemed to be to find if this effect might be

referred back to an interference with the effectiveness of insulin and further, whether the glycogenic function of the liver and tissues was in any way affected.

The effect of alkalosis on the action of insulin.

For this purpose rabbits and rats have been used. It will be unnecessary to give more than typical experiments. Similar results to my own have been obtained by Hetenyi [1926], who found that the effect of insulin is strongly delayed by alkali given *per os*. He also found that acid given *per os* had no appreciable effect on the action of insulin. The findings with alkali have been confirmed and we give the following experiment on a single rabbit, which was very striking.

This animal, which had been observed on several occasions, could have its blood-sugar lowered to values of 0·02 % without symptoms. As we had never seen such an animal before experiments were repeated several times with the following results.

Exp. 6. Effect of two units of insulin injected subcutaneously on the blood-sugar of the above rabbit. Weight 2 kg. There were no symptoms. No food, of course, was given during these experiments.

Time	2.25	2.40	3.10	4.15	4.45
Blood-sugar g. %	0·096	Insulin 2 units	0·062	0·020	0·020

Exp. 7. Same rabbit as above. Blood taken from veins of both ears.

Time ...	12.30	12.35	1.15	2.10	3.08	3.40	4.35	4.40
Blood-sugar g. % ...	0·135	Insulin 3 units	0·090	0·030	0·020	0·025	0·125	0·122

Exp. 8. In conditions similar to those of Exps. 6 and 7 the same rabbit was given 3·5 g. of $NaHCO_3$ by stomach-tube at 12 o'clock and insulin injected as below.

Time ...	11.20	12.00	12.08	12.55	1.35	2.20
Blood-sugar g. % ...	0·111	0·152	Insulin 4 units	0·177	0·131	0·122
Time ...	2.45	3.20	3.50	4.20	6.00	
Blood-sugar g. % ...	Insulin 4 units	0·072	0·056	0·067	No symptoms	

In this last experiment the initial rise of blood-sugar to 0·152 % was due to the passage of the stomach-tube. The first dose of insulin brought the blood-sugar down to 0·122 % in 2 hours 12 minutes. A further dose of four units only lowered it to 0·067 % in a further 1½ hours, a level which could be attained by two units in ½ hour starting at levels between 0·08 and 0·09 %. When it is considered that altogether eight clinical units were injected, it seems clear that the alkali has in some way definitely inhibited the action of the insulin.

It seems feasible to suppose that a similar sort of thing is occurring in the alkalosed human subject and that here lies the explanation of the intolerance to carbohydrate which accompanies alkalosis.

THE EFFECT OF ALKALOSIS ON GLYCOGEN STORAGE.

For this purpose white rats were used. The recent work of Macleod and co-workers [Barbour, Chaikoff, Macleod and Orr, 1927] on the glycogen distribution in rats under the influence of insulin is of great importance in showing how to obtain uniform controls in these animals. For our experiments, however, it soon became evident that absolutely uniform controls were unnecessary as the changes induced by alkali were remarkable.

White rats of between 130 and 140 g. weight were taken and put on a diet of bread and milk. This diet suits them admirably and good deposits of glycogen are soon laid down both in liver and muscles. After the required period of feeding the animals were weighed, tapped smartly on the head and immediately decapitated. The liver was quickly weighed and worked up for glycogen. The carcase was rapidly skinned, eviscerated, weighed and an aliquot part worked up for glycogen.

Exp. 9. Three rats on bread and milk. No glycosuria. No ketosis.

No.	Days on bread and milk	Weight (g.)	Weight of liver (g.)	Weight of skinned carcase (g.)	Glycogen (mg.)	
					Liver	Carcase
1	8	142	6·28	56	104	36
2	13	135	6·39	60	96	69
3	16	152	6·78	61·5	254	114

Exp. 10. Another four rats which had been on the same diet as those in Exp. 9 were now given $NaHCO_3$ added to their bread and milk. The periods of alkalisation varied from 3 to 8 days. The animals took the bicarbonate well and passed considerable amounts of alkali in the urine. In one case 0·49 g. of $NaHCO_3$ appeared and in another 0·66 g. in one day. The following table gives the results of analysis of these animals.

No.	Days on bread and milk	Days on alkaline diet	Weight (g.)	Weight of liver (g.)	Weight of skinned carcase (g.)	Glycogen (mg.)	
						Liver	Carcase
4	29	3	149	5·83	71	Traces	13
5	33	7	135	4·81	60	Traces	Traces
6	34	8	132	4·86	63	26	Traces

The fourth alkalosed rat was now taken off the alkali and put on an ordinary bread and milk diet for 2 days. On the first day after the return to ordinary diet it excreted alkali but no acetone and there were 48 mg. (as glucose) of Cu-reducing substances in the urine. The urine also reduced alkaline safranine so that sugar was probably present. On the second day the urine was amphoteric, acetone was present and the reduction value was 53 mg. The rat was now killed and analysed.

Exp. 11. Rat no. 7.

Days on alkalised diet	Days of return to ordinary diet	Weight (g.)	Weight of liver (g.)	Weight of skinned carcase (g.)	Glycogen (mg.)	
					Liver	Carcase
8	2	140	7·92	52	156	104

The above Exps. 9, 10 and 11 seem definitely to show that in conditions of powerful alkalosis the animals do not lay down glycogen but the readjustment to normal storage is rapid when the excess alkali is removed.

SUMMARY.

1. Utilising doses of $NaHCO_3$ up to 30 g. the maximum concentration of bicarbonate in the urine of a normal subject is about 3·2 %. The rise in bicarbonate excretion is constantly associated with a very marked fall in chloride excretion. The fall in chloride excretion is not accompanied by a fall in blood- or plasma-chloride. It is difficult to be sure whether this effect is due to an inability of the kidney to excrete chloride or to a diversion of tissue-chloride so that the "head" of chloride is removed.

2. The fall in urinary chlorides in alkalosis is not accompanied by a similar phenomenon in the stomach, where the secretion of chloride may be quite normal.

3. Alkalosis is accompanied by a definite diminution in carbohydrate tolerance and sometimes by a ketosis. The alkalosis-ketosis is not a mere "washing-out" effect, but is rather to be regarded as a result of defective carbohydrate utilisation.

4. The ketosis of starvation is much increased in alkalosis and the maximum excretion of ketonic acids does not necessarily coincide with the maximum output of alkali in the urine.

5. The ketolytic action of glucose in starvation is markedly diminished by alkali.

6. The action of insulin is diminished by alkali given *per os*.

7. The deposition of glycogen in liver and muscle can be greatly diminished by alkalosis.

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CXXXIII. A NOTE ON THE ESTIMATION OF ADRENALINE.

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(*Received June 30th, 1927.*)

Introduction.

During the progress of other work in this laboratory it was necessary to carry out quantitative estimations of the adrenaline present in the small adrenal glands of the rat and pigeon. The well-known colorimetric method of Folin, Cannon and Denis [1913], described for estimations on glands of about 2 g. in weight, seemed the most suitable for this purpose.

On adapting this method to the small glands of about 0·03 g. weight that were being used, results were obtained that showed considerably wider variation than was anticipated. At the same time it was noticed that, under the conditions of our adaptation, the rate of colour change of the adrenaline solution differed from that of the uric acid standard solution.

Accordingly it was decided to investigate the effect of such variable factors as temperature on the rate of colour production of solutions of adrenaline, using the glasses of a Lovibond tintometer as a standard against which to match the blue colour.

The possibility of loss of adrenaline due to oxidation in the method of extraction employed by Folin, Cannon and Denis was also examined and found to be small but definite. This loss takes place after the addition of sodium acetate to the hot hydrochloric acid extract and was avoided by using cold trichloroacetic acid as the extracting agent. This acid proved satisfactory in every way and gave a clear protein-free filtrate.

Apparatus.

The best source of light for use with a tintometer is daylight from a north window. However, as we wished to work at any time and during the winter months an artificial source of light was necessary. A stout cardboard box was painted black inside and placed upon its side, which was 42 cm. long. Two daylight lamps were inserted in the end, one above the other and 15 cm. apart, and across the other open end of the box two sheets of the special transparent paper supplied by the Tintometer Company, Salisbury, were placed close together. A third sheet of this paper was inserted about 18 cm. nearer the lamps

than the other two sheets. Such an arrangement gave a fairly large and evenly illuminated field and the tintometer was placed at such a distance that the structure of the transparent paper could not be seen. Two small weighing bottles, of internal diameter 2·35 cm., served as cells for holding the solutions to be matched. During the day both daylight lamps were employed but at night it was found easier to match if only one was used. A thermostat was maintained at 20° (or the appropriate working temperature) and held the matching cells and all the reagents and vessels used in producing the blue colour.

Temperature and other factors.

By using a tintometer the blue colour may be matched against a non-variable standard and the difficulty of matching two colours of not quite the same tint, such as those given by adrenaline and uric acid with the phosphotungstic "uric acid reagent," is avoided.

In order to plot time-intensity curves of the blue colour developed, an accurately weighed amount of adrenaline, between 0·03 and 0·04 g., was dissolved in 10 % trichloroacetic acid and the volume made up to 100 cc. with this acid. Small portions of this standard solution, between 1 cc. and 5 cc., were diluted to 25 cc. with 10 % trichloroacetic acid and 3 cc. of such a solution were run into a 25 cc. flask, diluted somewhat with water and placed in the thermostat. In this way the concentration of adrenaline could be readily varied while the acid concentration remained constant. When sufficient time had been allowed for the flask and its contents to reach the appropriate working temperature, 0·5 cc. of the phosphotungstic "uric acid reagent" described by Folin and Trimble [1924] was added, washed in with a little distilled water, the time noted, 5 cc. of a saturated solution of sodium carbonate added, washed in and made up to a final volume of 25 cc. with distilled water. The flask was then shaken briskly and a suitable amount of the solution poured into the matching cell. The blue colour was matched at measured intervals of time after the addition of the sodium carbonate. The amount of adrenaline estimated in these experiments was of the order of that present in 0·03 g. of adrenal gland. It was found that the blue glasses of the tintometer required the admixture of about 10 % of the yellow glasses in order to match the tint produced by adrenaline, but only the value of the blue glasses was recorded. It was difficult to match accurately any solution paler than five or six blue units.

The time-intensity curves for different concentrations of adrenaline were plotted at 20°. These curves, shown in Fig. 1, rise to a maximum in almost exactly 20 minutes and demonstrate clearly that, over the range studied, the concentration of adrenaline does not affect the time of maximum colour development at any one temperature.

The rate of colour development for one concentration of adrenaline at 14°, 16° and 25° was next studied. Fig. 2 shows the curves obtained; clearly, the reaction velocity is greater at higher temperatures, the time taken to reach a maximum increasing as the temperature falls; at 25° the maximum is reached

after 10 minutes, at 16° after about 35 minutes and at 14° after 45 minutes. The curves at 25°, 16° and 14° are typical curves selected from a number obtained at those temperatures, while the curve at 20° is an average of all those obtained for that concentration at that temperature.

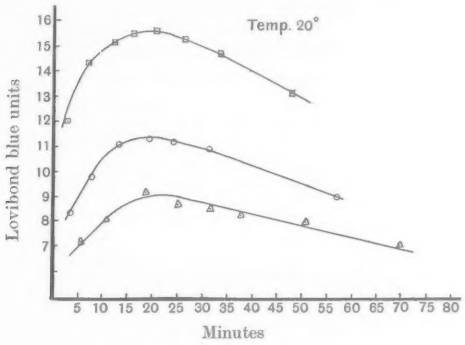

Fig. 1.

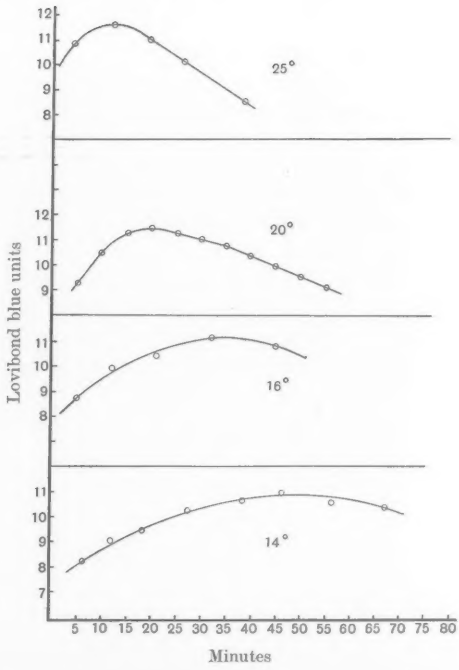

Fig. 2.

In order to estimate adrenaline in solutions of unknown strength, a calibration curve was constructed. Such a curve is of course dependent on the size of cell for matching used and possibly also on the particular specimen of "uric acid reagent" employed and must therefore be independently constructed by each worker. As a working temperature 20°, at which maximum colour develops in 20 minutes, was selected as being the most convenient. The known adrenaline content was plotted against the blue colour developed after 20 minutes. Three such points were taken from the curves in Fig. 1 and two more were obtained from separate determinations, one for a higher and the other for a lower concentration of adrenaline. The calibration curve is a mean straight line drawn through these points. Other known concentrations of adrenaline were then estimated and marked on the graph. From these the possible maximum experimental error was calculated to be 7 %.

Oxidation.

The possibility of loss of adrenaline by oxidation during the boiling in hydrochloric acid solution after addition of sodium acetate, in the method of Folin, Cannon and Denis, was investigated with solutions of pure adrenaline and, except where mentioned below, following their technique exactly. In each experiment about 0·008 g. of adrenaline was used, as this was a fair average of the amount estimated by these workers in 2 g. of adrenal gland. After addition of sodium acetate, in the first experiment the solution was brought to the boil, made up to 100 cc., roughly divided, one half cooled rapidly under the tap and the other half allowed to cool slowly on the bench. 1·25 cc. of one half was then pipetted into a 25 cc. flask and matched in the way described in the last section. 1·25 cc. of the other half was similarly estimated. In the second experiment boiling after addition of sodium acetate was continued for one minute and in the third experiment for two minutes; the subsequent procedure in these two experiments was the same as in the first.

Table I shows that no significant loss takes place if the method of Folin, Cannon and Denis be followed exactly, but that if the boiling be continued for 2 minutes and the solution allowed to cool slowly there is a 10 % loss. Thus when working with quantities of adrenaline a hundred times as small, appreciable loss might readily occur.

Table I.

Exp.	Treatment after addition of NaAc	Units of blue with cooling		Units of blue in control	Loss		% loss	
		Rapid	Slow		Rapid	Slow	Rapid	Slow
1	Brought to boil	12·4	12·0	12·6	0·2	0·6	2·0	5·0
2	Boiled for 1 min.	12·1	11·6	12·6	0·5	1·0	4·0	8·0
3	Boiled for 2 mins.	11·7	11·4	12·6	0·9	1·2	7·5	10·0

Extraction of adrenal glands.

The efficiency of a 10 % aqueous solution of trichloroacetic acid as an extracting agent was investigated. A number of fresh rat adrenals were available.

These were minced finely with scissors to give a fairly homogeneous mash and three samples were weighed out, two of about 0·03 g. and one of 0·06 g. The adrenaline content of one 0·03 g. sample was estimated directly and to each of the other two samples a volume of an adrenaline solution equal to that of a previously estimated volume from the same solution was added. The glands were then ground fine and the adrenaline content of each estimated. The results are set out in Table II. The very slight loss observed shows that trichloroacetic acid is a very suitable extracting agent.

Table II.

Exp.	Weight of gland in g.	Units of blue observed	Control blue units	Blue units adren. pres. calc. from 0·0315 g.	Units of blue calc., *i.e.* control + calc.	Loss blue units	% loss
1	0·0315	4·9	5	—	—	—	—
2	0·0329	9·9	5	5·1	10·1	0·2	2·0
3	0·0629	14·5	5	9·8	14·8	0·3	2·0

Our extracts were accordingly made in the following way. The adrenal glands, in which the adrenaline was to be estimated, were carefully freed from connective tissue and transferred to a tared pyrex test-tube cut down to half size; this was weighed again. A little purified sand and a few drops of about 10 % trichloroacetic acid solution were added and the gland was finely ground in the tube with a glass rod, 3 cc. of the 10 % trichloroacetic acid were added and the sand and precipitated protein agitated, the sediment was allowed to settle and the liquid filtered through a wad of cotton-wool into a 25 cc. flask. The residue in the tube was washed first with 5 cc. and then with 3 cc. of distilled water plus a few drops of trichloroacetic acid solution, the washings were filtered into the flask and the latter set in the thermostat. The extract was then made up and matched in the manner described in an earlier section. Table III shows some results for a number of normal male rats.

Table III. *Starved 6–8 hours before experiment.*

Rat	Body weight (g.)	Rect. temp. ° C.	Weight adrenal glands (g.)	per 100 g. body weight (g.)	Adrenaline mg. mg. adrenaline	Adrenaline mg. per 1 g. gland	Adrenaline mg. per 100 g. body weight
1	126	37·7	0·0386	0·0306	0·086	2·23	0·068
2	130	38·1	0·0334	0·0257	0·083	2·49	0·064
3	119	38·2	0·0338	0·0284	0·074	2·19	0·062
4	124	36·6	0·0376	0·0303	0·071	1·89	0·057
5	129	37·3	0·0320	0·0248	0·058	1·83	0·045
Average	126	37·6	0·0337	0·0267	0·074	2·13	0·059

The Authors wish to express their thanks to Prof. J. C. Drummond for much helpful advice and his constant interest in the work. One of them (G. F. M.) is indebted to the Medical Research Council for a personal grant.

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CXXXIV. THE CONSTITUENTS OF *FABIANA IMBRICATA*.

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(*Received June 20th, 1927.*)

The plant *Fabiana imbricata* (Ruiz and Pavon) or *pichi-pichi* is a well-known shrub growing in South America and belongs to the Solanaceae.

It has been used medicinally for centuries by the Incas, and about 50 years ago attracted some attention in Europe as a specific for urinary and liver complaints.

The first chemical examination appears to have been carried out by Nivière and Lotard [1887], who state definitely that the drug is free from alkaloid. They ascertained the presence of a fluorescent principle, a yellow precipitate with lead acetate analogous to "l'esculo-tannate de plomb," gummy matter, glucose and calcium tartrate.

A more detailed examination was carried out, however, by Kunz-Krause [1899], who found the leaves to contain choline, chrysotropic acid (now known as scopoletin) and a substance which he called "fabioglucotannoid" and for which he suggested the formula:

$$C_6H_{11}O_5.O.C_6H_2(OH)(OMe)CH : CH.CO_2H$$

or its internal anhydride. The compound was described as a yellow, very hygroscopic substance softening at 80° and beginning to swell up at 105°.

Kunz-Krause further isolated from the wood a volatile oil which he called fabianol (no formula given) together with a non-volatile white crystalline solid melting at about 280° and capable of being sublimed. This was called fabianaresen and the formula was $C_{54}H_{90}O_6$ although cryoscopic determinations indicated $C_{18}H_{30}O_2$.

It is evident that Kunz-Krause isolated products of doubtful purity, and the results obtained in the present investigation have confirmed this, more especially in regard to the so-called fabioglucotannoid.

This substance has now been shown to be a new glucoside of scopoletin for which the name *fabiatrin* is proposed, and its properties are described in the experimental part of this paper.

The authors have been unable to confirm the presence of choline, but have indicated the presence of a basic substance which forms a picrate. In addition, a saponin has been detected, and the physiological effect of the plant appears to be due entirely to this substance. Although the drug is classified among the Solanaceae, the absence of alkaloids is confirmed.

Experimental.

The material used in this investigation consisted of twigs about 3 feet in length and varying in diameter from 1 to 2 inches. The cortex was very thin, and the wood extremely hard. The drug possessed a characteristic odour, and a substance (alcoholic) was isolated possessing the odour in a marked degree.

The twigs were very kindly ground for us by Messrs Evans, Lescher and Webb, Ltd., and we would here express our deep appreciation to C. J. S. Sendall, Esq., for the help so readily given.

As a preliminary experiment, 50 g. of the ground material were successively extracted in a Soxhlet apparatus, when the following amounts of extract, dried at 100°, were obtained.

Light petroleum (B.P. 29–45°) extracted		0·44 %
Ether	,,	0·56 ,,
Chloroform	,,	1·40 ,,
Ethyl acetate	,,	1·07 ,,
Alcohol	,,	1·52 ,,
	Total	4·99 %

In order to glean some idea of the nature of the drug, two portions (500 g. each) were treated respectively with (*a*) sodium carbonate (30 g.) and (*b*) hydrochloric acid (2 %).

(*a*) On distillation in steam, 0·7 g. of an essential oil was obtained which was neutral, and contained no furfuraldehyde. The aqueous distillate was also neutral, thus proving the absence of volatile bases.

(*b*) In this case the acidic solution was filtered from the marc, and although the filtrate gave the usual alkaloidal reactions, only resinous products were obtained.

For the purpose of a complete examination of the drug, a quantity (5 kg.) of the ground material was thoroughly extracted with light petroleum, ether and alcohol.

Examination of the light petroleum extract.

After the removal of the solvent, there remained a dark green viscous mass amounting to 15 g. The residue was taken up in ether when, on keeping, a small quantity of sparingly soluble substance separated as a white amorphous powder. This was collected and washed thoroughly with ether when it was found to melt between 74° and 77°; and, after recrystallisation from ethyl acetate, it was obtained in colourless plates melting at 82° (found, C = 78·05 % and H = 12·7 %). On hydrolysis with alcoholic potash, an alcohol melting at 80° and an acid melting at 77·5–78° were obtained. The above compound is evidently an ester, and in view of the subsequent isolation of arachidic acid, it is very probable that the acidic portion of the ester is also arachidic acid.

The ethereal liquid, after the removal of the above-described ester, was extracted with solutions of ammonium and sodium carbonates, and 5 % sodium hydroxide.

The ammonium carbonate removed a mere trace of substance.

The sodium carbonate removed 0·1 g. of an acid which, when recrystallised from ethyl acetate, melted at 62°. This acid was probably palmitic acid, but the amount obtained was too small to confirm by analysis.

The sodium hydroxide also removed a further small quantity of the acid melting at 62°.

The ethereal liquid, containing the neutral portion of the extract soluble in light petroleum was finally dried over anhydrous sodium sulphate and the solvent removed, when there was left a quantity of viscous product. This was hydrolysed with alcoholic potassium hydroxide, and worked up in the usual manner for the isolation of the unsaponifiable constituents and the combined fatty acids. In this way there were isolated (*a*) a phytosterol (M.P. 134°) which gave an acetyl derivative melting at 117°; (*b*) a *new* unsaturated alcohol (B.P. 145°/25 mm.) agreeing with the formula $C_{26}H_{42}O$ (found: C = 84·6 %; H = 11·3 %. Calc. for $C_{26}H_{42}O$: C = 84·8 %; H = 11·3 %). This alcohol possesses the characteristic odour of the drug and appears to be the odoriferous principle; it gave the colour reactions of the phytosterols; (*c*) traces of unsaturated fatty acid and (*d*) arachidic acid (M.P. 77°). (Found: C = 76·6 %; H = 13·0 %. Calc. for $C_{20}H_{40}O_2$: C = 76·9; H = 13·0 %.)

Examination of the ethereal extract.

This was a dark green soft solid amounting to 20 g. It gave only (*a*) a grey amorphous solid (M.P. 86°) and (*b*) a colourless crystalline substance (M.P. 82°), both being insufficient to identify. There was a complete absence of any glucoside in this extract.

Examination of the alcoholic extract.

This extract consisted of a dark coloured resin amounting to 150 g. It was mixed with water, and the mixture distilled in a current of steam. The distillate contained a small amount of volatile oil, but nothing acidic or basic. After the above operation, there remained in the flask an orange-coloured aqueous liquid together with a quantity of dark brown resin. The latter amounted to 40 g., from which nothing definite was isolated.

Examination of the aqueous liquid.

This liquid, after being separated from the resin, was concentrated *in vacuo* to about one litre and repeatedly extracted with ether.

Isolation of scopoletin, $C_{10}H_8O_4$. The ethereal extracts were united and concentrated to small volume, when, on keeping, 7 g. of a pale yellow solid separated. This was collected and recrystallised from alcohol, giving pale yellow needles (M.P. 204°), and a brilliant blue fluorescence with alkalis. The

substance was identified with scopoletin. (Found: C = 62·5 %; H = 4·18 %. Calc. for $C_{10}H_8O_4$: C = 62·5 %; H = 4·2 %.)

An *acetyl* derivative was prepared which separated from alcohol in slender colourless needles, M.P. 177°. (Found: C = 61·25 %; H = 4·4 %. Calc. for $C_{10}H_7O_4.COCH_3$: C = 61·5 %; H = 4·3 %.)

After washing the ethereal solution and drying, 0·1 g. of a pale yellow solid separated on keeping for a short time. It was found to be phenolic in character, but it was quite amorphous and had no definite melting point.

Isolation of a basic principle. The original aqueous liquid, after being extracted with ether as described above, gave precipitates with the usual alkaloidal reagents and possessed a bitter taste. A portion of the solution was therefore made alkaline with sodium carbonate and repeatedly extracted with ether. The ethereal extracts were united, dried and the solvent removed, when there remained a small quantity of a gummy mass. The product contained nitrogen and gave reactions for an alkaloid. It could not be obtained crystalline. A solution in hydrochloric acid gave a brown amorphous product with mercuric chloride, and a *picrate* was readily formed which was soluble in hot alcohol, but separated in the amorphous state, melting with decomposition at 125°. No crystalline derivatives could be prepared.

The remainder of the aqueous liquid was concentrated *in vacuo* to a syrup, and a small portion of the latter was treated with phenylhydrazine acetate, when an osazone separated. This was recrystallised from dilute pyridine, when it melted at 210° thus being identified as *d*-phenylglucosazone.

Isolation of a hitherto undescribed glucoside of scopoletin. The concentrated aqueous liquid was mixed with a quantity of absolute alcohol when a precipitate separated. This redissolved on warming and again separated on cooling as a brown solid.

After filtration the substance was recrystallised from dilute alcohol using a little charcoal to remove colouring matter, when it separated in rosettes of needles melting at 226–228°.

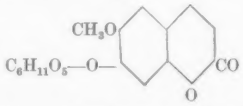
The compound gave scopoletin and glucose on hydrolysis with dilute acid, and analysis proved it to be the glucoside of scopoletin having the formula $C_{16}H_{18}O_9$, $2H_2O$. Dried at 100°. Found: C = 48·7 %; H = 5·6 %. Calc. for $C_{16}H_{22}O_{11}$: C = 49·2 %; H = 5·6 %. Dried at 130°. Found: C = 51·2 %; H = 5·35 %. Dried at 160°. Found: C = 51·27 %; H = 5·3 %. Calc. for $C_{16}H_{20}O_{10}$: C = 51·6 %; H = 5·3 %.

It was found impossible to remove the second molecule of water, and a separate determination of the substance dried at 160° showed a loss of 4·6, the calculated amount for $1H_2O$ being 4·8 %.

The above glucoside has not been described hitherto in the literature and it is proposed to designate it *fabiatrin*.

Fabiatrin is a colourless solid which crystallises in needles, readily soluble in hot water, but very sparingly soluble in the usual organic solvents in the cold. The aqueous solution gives a yellow colour with ammonia and caustic

soda, indicating the opening of the pyrone ring, and the constitution of the glucoside may be represented thus:



Isolation of a saponaceous substance. The dilute alcoholic solution of the syrup, after the removal of the glucoside, was concentrated to small volume and kept for some days. A further quantity of fabiatrin separated, the total amount obtained being 7 g. After removal on the filter, the filtrate was digested with a quantity of absolute alcohol, when a brown amorphous solid separated, whose properties indicated that it belonged to the saponins. A very dilute aqueous solution produced a foam which remained stable for many days. It possessed an exceeding bitter taste, but in the dry state was not sternutatory.

Physiological tests.

These test were kindly conducted by Prof. W. J. Dilling and the results (indicated briefly) show that the drug is poisonous to animals, a decoction having proved fatal to frogs, rabbits and cats. The poisoning effect appears to be due to a saponin, and such a substance has been shown to be present.

A separate examination of scopoletin has shown this substance to be quite inert, both physiologically and bacteriologically—the latter experiments being kindly done by Prof. J. M. Beattie.

The physiological action of fabiatrin will be described in due course.

Summary.

A complete examination of the aerial stems of *Fabiana imbricata* (Ruiz and Pavon) has been conducted, and the following substances have been isolated. A neutral essential oil which gave a negative reaction for furfuraldehyde; an ester (M.P. 77·5–78°); palmitic acid; a phytosterol (M.P. 134°); a new unsaturated *alcohol*, $C_{26}H_{42}O$ (B.P. 145°/25 mm.), possessing the fragrant odour of the drug; traces of unsaturated acids; arachidic acid; scopoletin; a basic compound which gave an amorphous picrate (M.P. 125° with decomposition); a sugar giving *d*-phenylglucosazone; a new glucoside of scopoletin, $C_{16}H_{18}O_9.2H_2O$ (M.P. 226–228°), designated *fabiatrin*; and an amorphous substance possessing the properties of a saponin.

In conclusion, the authors express their warmest thanks to Prof. I. M. Heilbron for suggesting the research; to Dr C. J. MacAlister, F.R.C.P., for his instrumentality in providing the material, and also a grant towards the expenses of the investigation; and to Profs. Dilling and Beattie for conducting the physiological and bacteriological tests.

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CXXXV. VARIATIONS IN THE SERUM-CALCIUM OF RABBITS.

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(*Received June 21st, 1927.*)

In a comparison of the effect on rabbits of the parathyroid hormone with that of insulin and various other substances, Davies, Dickens and Dodds [1926] showed that the serum-calcium could be raised by the injection of insulin at regular intervals of time. The curve that they obtained for the serum-calcium resembled those published by Clark and Collip [1926, 2] showing the results of similar repeated injections of parathyroid extract into dogs. Further, by following Clark and Collip's [1926, 1] method of extraction of the parathyroid hormone, they obtained a product from pancreas which, when injected into rabbits, caused an increase in the serum-calcium; but both with this extract and that of the actual parathyroid glands a rise was only obtained when very large doses were used. The authors suggested that the parathyroid hormone, like insulin, might be fairly widely distributed in the body. If these two substances occur together in the pancreas, owing to the marked similarity between their properties they might be extracted together and some of the active principle of the parathyroid would then remain as an impurity in the final product of insulin. Hence, the effect observed on the serum-calcium of the rabbit might be due, not to some additional property of insulin, but to its association with this impurity.

Should this hypothesis prove to be correct, a number of practical results would follow, and it appeared worth testing with regard to the possibility of obtaining (*a*) a new criterion for the purity of insulin—that it should have no effect on the serum-calcium; (*b*) a use for apparently worthless by-products of insulin manufacture; and (*c*) an explanation of the frequent very erratic response of rabbits to insulin. While the hypoglycaemic effect is known to last for only a few hours, that on the calcium might be much more prolonged and might account both for the "bulk variation" of groups of animals in their reaction to insulin, which has been repeatedly observed by workers engaged in its physiological assay, and also for the individual variations of particular animals.

Method.

A group of ten young rabbits was selected for experiment. Four acted as controls throughout, two receiving doses of saline, two having no injections at all.

All doses were calculated on the weight of the animal, and dilutions were made such that a volume of 0·25 cc. per kg. was always injected. The animals

were fed throughout unless otherwise stated, their diet consisting of hay, oats and cabbage.

Serum-calcium determinations were carried out by the method of Kramer and Tisdall [1921] modified by Clark and Collip [1926, 3].

To test the method, seven separate estimations of serum-calcium were made on the same blood and gave the following values in mg. per 100 cc. of serum: 15·37, 15·27, 15·18, 15·18, 15·27, 14·88, 15·08. Hence, a difference of 0·5 mg. per 100 cc. can be regarded as outside the range of experimental error.

The effect of insulin on the serum-calcium.

Since Davies, Dickens and Dodds observed a significant rise four hours after the injection of what they describe as a sample of "commercial insulin," a number of preliminary experiments were made in which blood samples were taken before and four hours after injection; but neither with the purest nor with very impure samples of insulin was any rise obtained, and both the rabbits receiving insulin and the controls which received none gave similar results.

The following is a typical experiment.

The rabbits were given a dose of one unit per kg. of a very impure preparation of which one unit was contained in 8 mg. They had received their morning ration of cabbage about half an hour before the experiment was started, and hay and oats were present in their cages throughout. The results are shown in Table I, and, for the sake of comparison, figures are also given for an experiment using the same animals in which precisely similar conditions were observed but no injections were given to any of them.

Table I.

		(1) Nov. 10th			(2) Nov. 17th	
		Serum-calcium mg. per 100 cc.			Serum-calcium mg. per 100 cc.	
Rabbit no.	Dose	Initial	4 hours	Dose per kg.	Initial	4 hours
N 1	None	14·50	13·82	1 u.	15·09	13·44
N 2	,,	14·31	13·44	,,	14·31	12·96
N 3	,,	17·12	14·60	,,	14·89	13·54
N 4	,,	14·89	13·73	,,	14·41	14·41
N 6	,,	15·57	14·21	,,	14·50	14·02
O 6	,,	16·44	13·85	,,	13·82	12·47
Average		15·47	13·94	—	14·50	13·47
Change from initial...		—	−1·53	—	—	−1·03
Control 1. No insulin. Saline.						
O 1	None	13·82	13·35	0·25 cc.	14·01	11·70
O 10	,,	14·79	13·24	,,	13·93	12·86
Average		14·31	13·30	—	13·97	12·28
Change from initial...		—	−1·01	—	—	−1·69
Control 2. No insulin. No saline.						
P 9	None	15·09	13·93	None	14·12	13·56
P 10	,,	15·18	13·68	,,	13·73	12·67
Average		15·14	13·81	—	13·93	13·12
Change from initial...		—	−1·33	—	—	−0·81

It will be seen that in both cases a fall was obtained. Work by Clark [1920], Moritz [1925] and Stewart and Percival [1927] suggests that this is the usual effect of haemorrhage on rabbits and a number of results obtained in the course of this investigation lead to the same conclusion. Haemoglobin estimations indicate a small but progressive dilution of the blood with respect to this constituent when rabbits are bled at frequent intervals of time. The two observations may possibly be correlated. In the above experiments, however, the animals were fed, and, as will be shown later, this may have a considerable effect: it is possible that with careful control of the diet the rabbit might show the same constancy with regard to the calcium content of its serum as has been demonstrated in the case of the cat (Stewart and Percival).

There appeared to be two possible explanations of these negative results:

(*a*) that the particular insulin samples used would cause a rise in serum-calcium at some other interval of time than that investigated in these preliminary experiments; or

(*b*) that only certain rabbits show this effect.

A. (i) *The effect of repeated small doses of insulin when rabbits are fed with hay, oats and cabbage.*

A number of experiments were performed in which the rabbits were given injections of 0·5 or 1 unit per kg. every two hours for six hours, blood samples being taken either at the 4th or 5th and 20th hours, but in no case was any rise in the serum-calcium obtained. Finally the curve for the whole 24 hours was followed, injections being made every four hours, and the conditions described by Davies, Dickens and Dodds being as far as possible observed.

According to the usual routine of this laboratory the rabbits were given hay, oats and cabbage at 9 a.m. and 5 p.m., and in addition during the night cabbage and oats were given at 3 a.m.

The results are shown in Fig. 1.

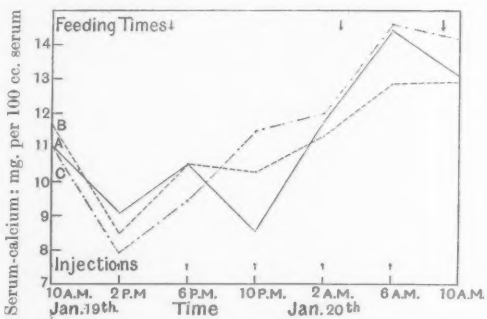

Fig. 1. A. Average curve for six rabbits receiving doses of 0·5 unit of insulin per kg. every four hours.

B. Average curve for two rabbits receiving doses of 0·25 cc. per kg. of normal saline solution every four hours.

C. Average curve for two rabbits having no injections.

Both the controls and the rabbits receiving insulin showed a preliminary fall followed by a rise to a high value. This rise cannot be due to insulin because the animals that received none reacted similarly. It is a significant fact that on every occasion on which cabbage was given there was a considerable increase. An apparent exception to this is seen in the rise at 2 a.m., but a probable explanation lies in the fact that the cabbage given at 5 p.m. was not all consumed until much later; thus, it was observed at 10 p.m. that all the rabbits on insulin and one of the controls still had cabbage in their cages left over from the 5 o'clock meal. Unfortunately no note was made of this at the time nor observation made on the other three controls, because the importance of these details of feeding was not then recognised. During the next four hours all the cabbage was eaten, since none remained in the cages at 2 a.m. Throughout the whole period oats and hay were present in all the cages, only the cabbage varied.

One rabbit went into convulsions at 9 o'clock and was given a large quantity of cabbage in the hope that it might recover without the necessity of an injection of glucose. It recovered without sugar and showed the greatest rise in serum-calcium.

A. (ii) *The effect of repeated small doses of insulin when rabbits are fed with hay and oats only.*

Fig. 2 shows the results of four-hourly doses of 0·5 unit per kg. of the same sample of insulin as was used in the above experiment, the rabbits receiving their usual diet but with the cabbage omitted.

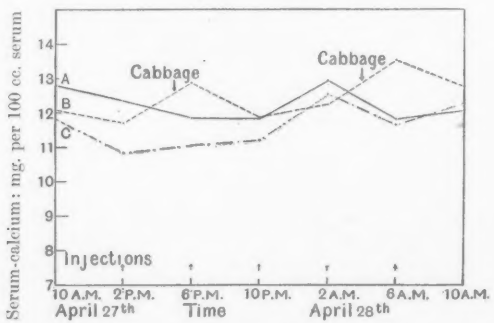

Fig. 2. A. Average curve for six rabbits receiving doses of 0·5 unit of insulin per kg. every four hours when cabbage was withheld.

B. Average curve for two rabbits receiving no injections but to whom cabbage was given twice during the experiment.

C. Average curve for two rabbits who received no insulin and no cabbage.

Cabbage was withheld from 12 noon on the day preceding experiment and water given instead until 5 o'clock in the evening in order to avoid any

possible complications from undue thirst, cabbage being normally the chief source from which these animals obtain water.

Of the four controls which received no insulin, two were given cabbage twice during the course of the experiment, at 5 p.m. and at 4 a.m. Both showed a rise in the serum-calcium at 6 p.m. and 6 a.m. It will be seen that the largest deviations from the initial values are shown by these two and that no changes in the serum-calcium of the animals that were injected can be ascribed to their doses of insulin.

B. *The effect of insulin convulsions.*

It is noticeable in the experiment reported by Davies, Dickens and Dodds in which they gave about 0·5 unit per kg. of insulin that the rabbit went into convulsions twice during the 24 hours in spite of the fact that it was fed throughout on cabbage and bran-mash. This led to the consideration that possibly only very sensitive rabbits show a rise in serum-calcium following insulin injection, and that actual convulsions might even be the cause. Hence, three very sensitive rabbits were selected which had been used for some time for insulin testing and were known to convulse on a dose of 0·5 unit per kg. when starved, and a series of experiments was performed with these animals (Table II). Again the results point to the conclusion that when a rise was observed it was caused by cabbage.

Table II.

Date	Conditions of experiment	Dose of insulin in units per kg.	Serum-calcium. Average change in 4 hrs after injection, in mg. per 100 cc.
Nov. 3, 1926	Fed at 9 a.m. with oats, hay and cabbage. Injected at 10 a.m.	0·5	−1·11
Nov. 8	Fed at 9 a.m. with oats, hay and cabbage. Injected at 10 a.m.	1·0	−0·56
Nov. 24	Starved for 22 hours. Injected at 10 a.m. All convulsed within 2 hours and were given sugar and cabbage	1·0	+1·32
Dec. 1	Starved for 22 hours. Injected at 10 a.m. All convulsed within 2 hours and were left in convulsive state for 1½ hours before being given sugar and cabbage	1·0	+1·26
Dec. 8	Starved for 22 hours. Injected at 10 a.m. and fed with hay, oats and cabbage at 10.30 a.m. in order to avoid convulsions	1·0	+1·10
Dec. 15	Ditto. (N.B. One convulsed in spite of feeding and was given sugar)	2·0	+1·26
Dec. 29	Starved for 22 hours. Initial blood samples taken at 10 a.m. Given oats, hay and cabbage at 10.30 a.m.	None	+1·26
Jan. 5, 1927	Starved for 22 hours and throughout experiment	None	−0·03
Jan. 12	Starved for 22 hours. Initial blood samples taken at 10 a.m. 6 cc. 20 % glucose injected subcutaneously at 1 p.m.	None	+0·52

0·5 and 1 unit per kg. failed to effect any increase in serum-calcium or to produce convulsions when the rabbits were fed. After starvation for 22 hours

(according to the usual routine for insulin testing) a rise was obtained with 1 unit per kg., both after convulsions when sugar and cabbage were given and also when convulsions were avoided by feeding the animals with hay, oats and cabbage half an hour after injection. The rise obtained could not be increased by withholding food and leaving the animals in convulsions for an hour and a half nor by doubling the dose. A control experiment in which the rabbits were fed with hay, oats and cabbage half an hour after the initial blood sampling but were given no injections confirmed the suspicion that insulin was not the cause of the rise obtained in the earlier experiments.

It is a routine in this laboratory that when glucose is used as an antidote to insulin convulsions the rabbit is also given a few cabbage leaves, the consumption of which is taken as a sign of recovery. Thus, on every occasion when the injection of sugar was necessary, the animals were also fed with cabbage.

An examination of the results shows that a rise was obtained after starvation in the following circumstances:

1. On feeding with cabbage and oats.

2. On feeding with cabbage and oats after the injection of insulin.

3. On feeding with cabbage and injecting sugar after the onset of insulin convulsions. (Sugar itself has not been shown to have any effect on the serum-calcium, either by us or by Davies, Dickens and Dodds.)

The only factor that did not vary is the consumption of cabbage. On no occasion was a significant rise obtained when cabbage was not given.

The effect of cabbage on the serum-calcium.

The conclusion having been reached by two separate series of experiments that any rise in serum-calcium following insulin injection is caused by cabbage and not by the insulin, the effect of cabbage alone was then tested.

The following procedure was followed.

At 12 noon on the day preceding experiment cabbage was removed from the cages and the animals were given water until 5 p.m. In order to control any possible effect of the water two of the controls were given none. Samples of blood were taken at 10 a.m., and six rabbits were then given a plentiful supply of cabbage which was maintained for 2 hours and then removed. The last four rabbits as usual served as controls and received no cabbage. All were kept supplied throughout with oats and hay, and serum-calcium determinations were made at two-hourly intervals. The results are shown in Fig. 3.

The only rabbit receiving cabbage which failed to show an increased serum-calcium ate none (*N* 6). For the purpose of averages it is therefore included among the controls. Those animals that were given no cabbage showed a slight fall.

Table III.

Exp 1.	Feb. 9th. Cabbage at 10 a.m. Serum-calcium mg. per 100 cc.				*Exp* 3.	April 6th. Cabbage at 11.30 a.m. Serum-calcium mg. per 100 cc.			
Rabbit no.	10 a.m.	12 noon	2 p.m.	4 p.m.	Rabbit no.	9.30 a.m.	11.30 a.m.	1.30 p.m.	3.30 p.m.
N 1	13·44	15·38	14·31	14·12	*U* 1	13·24	13·04	15·53	14·64
N 2	13·93	15·38	14·03	13·05	*U* 2	14·34	13·14	15·24	14·24
N 3	14·03	14·79	13·93	14·31	*U* 3	14·44	13·84	15·93	14·34
N 4	13·54	14·31	13·35	13·24	*U* 4	14·64	13·94	15·24	13·94
**N* 6	(13·24	12·76	12·76	12·38)	*U* 5	13·64	11·05	14·94	13·54
O 6	11·99	12·57	12·09	10·47	*U* 6	14·44	13·84	15·14	14·04
Average	13·39	14·49	13·54	13·04		14·12	13·14	15·34	14·12
Change from initial		+1·10	+0·15	−0·35		—	−0·98	+1·22	—
Control 1. No cabbage. Water given on the day preceding experiment.									
O 1	12·57	12·38	11·70	11·21	*U* 7	13·04	12·94	13·04	12·05
O 10	12·47	12·47	11·99	11·90	*U* 8	13·54	12·15	13·04	12·35
Average including *N* 6	12·76	12·54	12·15	11·83		13·29	12·55	13·04	12·20
Change from initial		−0·22	−0·61	−0·93		—	−0·74	−0·25	−1·09
Control 2. No cabbage. No water.									
P 9	13·15	12·86	12·18	11·12	*U* 9	14·24	13·84	12·84	13·04
P 10	12·47	13·35	11·99	11·21	*U* 10	13·14	13·14	11·65	11·55
Average	12·81	13·11	12·09	11·17		13·67	13·49	12·25	12·30
Change from initial		+0·30	−0·72	−1·64		—	−0·20	−1·44	−1·39

* *N* 6 ate no cabbage and is therefore omitted from the average and included in Control 1.

This experiment (Exp. 1, Table III) appeared to prove definitely that the previous conclusion as to the effect of cabbage was correct, and further confirmation was obtained by an experiment on the three abnormally sensitive rabbits which gave the same result.

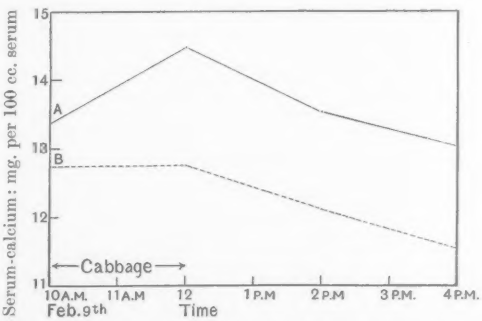

Fig. 3. A. Average curve for five rabbits fed with cabbage between 10 a.m. and 12 noon.
B. Average curve for five rabbits who were given no cabbage.

The next step attempted was to determine what constituent of cabbage has the property of raising the serum-calcium. For the purpose of making a systematic investigation of this problem it was thought advisable to start off with an entirely new set of rabbits. As a preliminary experiment, therefore,

the above test was repeated on these young rabbits. The results were entirely negative, the experimental animals reacting in the same way as the controls.

It is a well-known fact reported by many different observers that when the serum-calcium is high it is not easily changed, whereas when it is low it may be increased by a variety of means. Also it has been stated by Grant and Gates [1924], and confirmed by this work, that rabbits show a seasonal variation in serum-calcium, giving minimum values in January and high values in May and November. This is shown in Fig. 4, which gives a curve for the average of the initial values for ten rabbits during 3 months. Hence, the possible explanation of the different response to cabbage of the young rabbits tested in the middle of March from that of the group tested 5 weeks earlier is that the new ones were very near their maximum at the beginning of the experiment. Therefore, when it was repeated again on the same young animals 3 weeks later, instead of cabbage being given immediately after the initial blood samples were taken, it was given 2 hours later when the calcium content of the serum had already fallen. This time all the rabbits receiving cabbage showed a considerable rise (Fig. 5). The results are shown in Exp. 3, Table III.

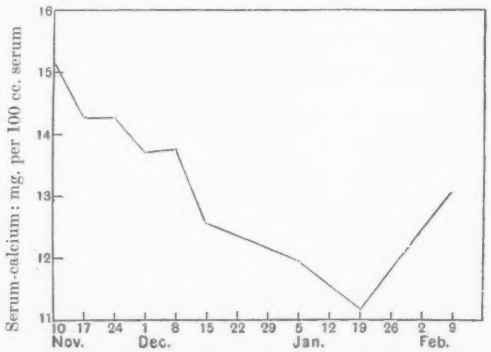

Fig. 4. Curve showing the average "seasonal variation" of the initial serum-calcium values for ten rabbits over a period of three months.

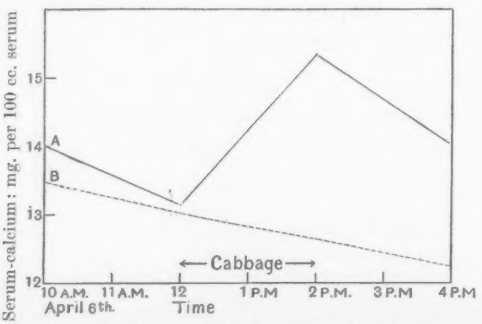

Fig. 5. A. Average curve for six rabbits fed with cabbage between 12 and 2 p.m.
B. Average curve for four rabbits who were given no cabbage.

Conclusions.

1. Insulin causes no rise in the serum-calcium of rabbits.
2. Insulin convulsions cause no rise in the serum-calcium of rabbits.
3. An immediate rise in the serum-calcium of rabbits occurs on feeding with cabbage, except when the initial value may be regarded as exceptionally high. The cause of this effect remains to be found.

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PAGE

CXXXI. The Toxicity of Phenylarsinic Acids for *Balantidium coli* Malm. in Cultures, in Relation to their Chemical Constitution. By A. S. Corbet and A. P. Jameson . . . 986

CXXXII. The Effect of Alkalosis on the Excretion of Chloride and on Carbohydrate Metabolism. By M. W. Goldblatt 991

CXXXIII. A Note on the Estimation of Adrenaline. By L. C. Baker and G. F. Marrian. (With two figures) 1005

CXXXIV. The Constituents of *Fabiana imbricata*. By G. R. Edwards and H. Rogerson 1010

CXXXV. Variations in the Serum-calcium of Rabbits. By K. Culhane. (With five figures) 1015

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